Evolution of HIV-1 subtype C immune responses during acute and chronic HIV infection

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CONTENTS

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ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ACD	Acid Citrate Dextrose
CEF	Cytomegalovirus (CMV), Epstein Barr Virus (EBV) and influenza virus (Flu)
CTLs	Cytotoxic T Lymphocytes
CV	Coeffiecient of variation
DMSO	Dimethyl Sulphoxide
EC	Elite controllers
EDTA	Ethylenediaminetetraacetic acid
ELISpot	Enzyme-linked immunosorbant spot
FBS	Foetal bovine serum
Gag	Group specific antigen
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HAART	Highly active antiretroviral treatment
IAVI	International AIDS Vaccine Initiative
IFN-g	Interferon gamma
ICS	Intracellular Cytokine Staining
LTNP	Long-term non-progressor
MHC	Major Histocompatibility Complex
Nef	Negative factor

PBMCs Peripheral blood mononuclear cells

PBS Phosphate Buffered Saline

PHA Phytohaemagglutinin

PVDF Polyvinylidene Diflouride

R 1 1% FBS in RPMI 1640 containing 50U penicillin, 50mg/ml streptomycin, 50mg/ml glutamine and 0.8mg/ml Fungin

R 10 10% FBS in RPMI 1640 containing 50U penicillin, 50mg/ml streptomycin, 50mg/ml glutamine and 0.8mg/ml Fungin

R 20 20% FBS in RPMI 1640 containing 50U penicillin, 50mg/ml streptomycin, 50mg/ml glutamine and 0.8mg/ml Fungin

SFU Spot-forming units

SIV Simian Immunodificiency Virus

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ABSTRACT

Background**.** Emergence of HIV-specific CD8 T cells during acute HIV infection is associated with significant reduction in HIV viral loads in plasma, restoration of CD4 T cell numbers in blood and resolution of the clinical symptoms associated with acute disease. Recent studies have shown that HIV-specific CD8 T cells that recognize conserved proteins such as Gag during both acute and chronic HIV infection are associated with better control of HIV replication. Unraveling the events during early infection that are associated with initial control of HIV replication and viral load may provide clues to the useful targets for development of efficacious therapy. The aim of this study was to compare the magnitude and breadth of HIV-specific T cell responses to HIV Gag and Nef mounted during acute HIV infection with those that emerged during chronic infection and to investigate the association of these responses with subsequent HIV disease progression (CD4 counts and plasma viral loads).

Method. Seven acutely HIV-infected women were enrolled into the acute HIV infection study at a mean of 21 days post-infection in collaboration with the Desmond Tutu HIV Foundation. In addition, nine long term non-progressors (LTNPs) who had been HIV infected for more than five years and maintained CD4 counts >350 in the absence of HAART were included for comparison. In both acute and LTNPs, the magnitude and breadth of IFN-γ T cell responses to HIV-1 subtype C Du422 Gag and/or consensus Nef overlapping peptides were assessed longitudinally by IFN-g ELISPOT. CD4 T cell counts and HIV-1 RNA loads in blood were measured at every visit.

Results. In women who were acutely infected with HIV, blood CD4 T cell counts were inversely associated with plasma HIV-1 RNA loads. In contrast, no correlation was found between CD4 T cell counts and plasma viral loads in LTNPs. The breadth of HIV-specific responses to both Gag and Nef peptide pools was narrow and responses were lower in magnitude during acute HIV infection compared to responses detected in the same individuals during chronic HIV infection. More women had detectable HIV Gag responses during acute infection than Nef although responses to Nef were generally higher in magnitude. IFN-g responses in LTNPs preferentially targeted the highly conserved p24 region of Gag. While the magnitude of T cell responses to Gag (particularly the p24 region of Gag) in the LTNP women was significantly inversely associated with plasma viral loads at the same time point; no correlation was observed in individuals who were acutely infected with HIV. No association was observed between the breadth of HIV-specific T cell responses and plasma RNA viral loads.

Conclusion. In this study, the magnitude and breadth of the Gag and Nef-specific responses shortly after HIV infection were not associated with initial control of HIV replication (although the cohort was very small). Both the magnitude and breadth of HIV-specific responses increased with time of infection. In contrast, HIV-specific responses in LTNPs were considerably stable over time.

CHAPTER 1

Literature Review

CHAPTER1

LITERATURE REVIEW

INTRODUCTION

1.1 Overview of the HIV-1 epidemic

From recent surveys, there are an estimated 33.3 million people infected with human immunodeficiency virus (HIV) globally (UNAIDS, 2010). An estimated 2.6 million new HIV infections were reported in 2009, which was 21% fewer than the highest incidence of 3.2 million reported in 1997 (Figure 1.1). The majority of the countries that recorded a lower rate in new infections were in Sub-Saharan Africa. This region still bears an alarming proportion of the global epidemic, however, accounting for 68% of people living with HIV. The UNAIDS report (2010) has cited that this decline in incidence is due to the advances in anti-retroviral therapies and efforts of improving access to treatment. The risk of HIV-1 transmission per heterosexual contact is high in low income countries (Boily *et al*., 2009). In contrast, an unprecedented resurgence of new infections were evident in several high income countries in Eastern Europe and Central Asia (Figure 1.2), and these were predominantly among men who have sex with men and in intravenous drug users and their sexual partners (UNAIDS, 2010).

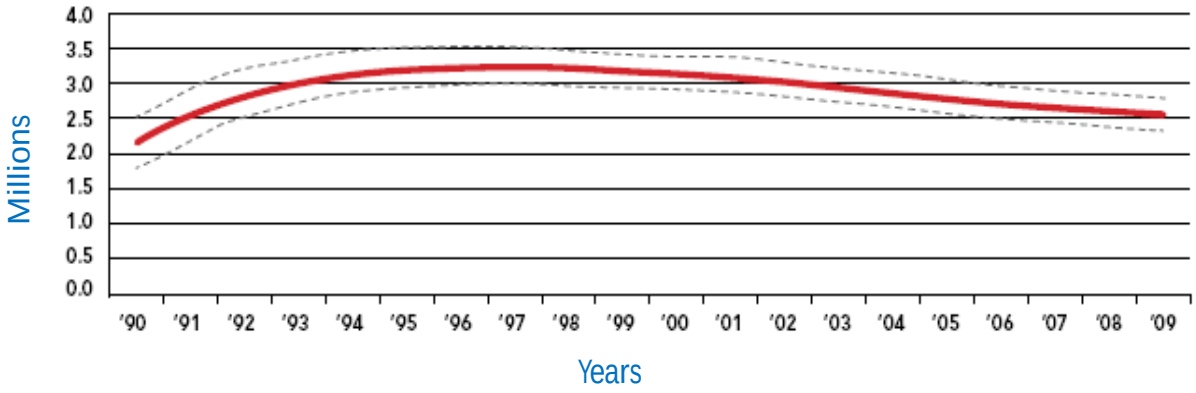

Figure 1.1 The total number of individuals globally who were newly infected with HIV. This graph shows the course of estimated incidence over a period of 20 years. The red line indicates the new estimates in millions while the dotted line indicates the plausible bounds (taken from UNAIDS, 2010).

South Africa is one of the countries that showed a decline in HIV incidence in the most recent UNAIDS report (2010) with a reported 25% decrease in new infections in 2009 compared with

2001 (Figure 1.2). South Africa, however, still has highest number of people in the world living with HIV with an estimated 5.2 million people infected. Furthermore, the prevalence in females within the age group 15-29 is almost 3–fold higher than the men in the same age group (RSA HIV/AIDS Progress Report, 2010; Figure 1.3). It has been speculated that the reason for the elevated incidence in young women compared to young men in South Africa is due to intergenerational sexual behavioral patterns where the women are much younger than their male sexual partners, making it more likely that these younger females are defenseless when negotiating the use of condoms in their older male partners (Parker *et al*., 2007; Shishana *et al*., 2009; Mercer *et al*., 2009). The recent success by a South African research team showing the 39% efficacy of a 1% Tenofovir-based microbicide gel has provided optimism and empowerment to young women who now have the ability to control prevention against HIV (Abdool-Karim *et al*., 2010).

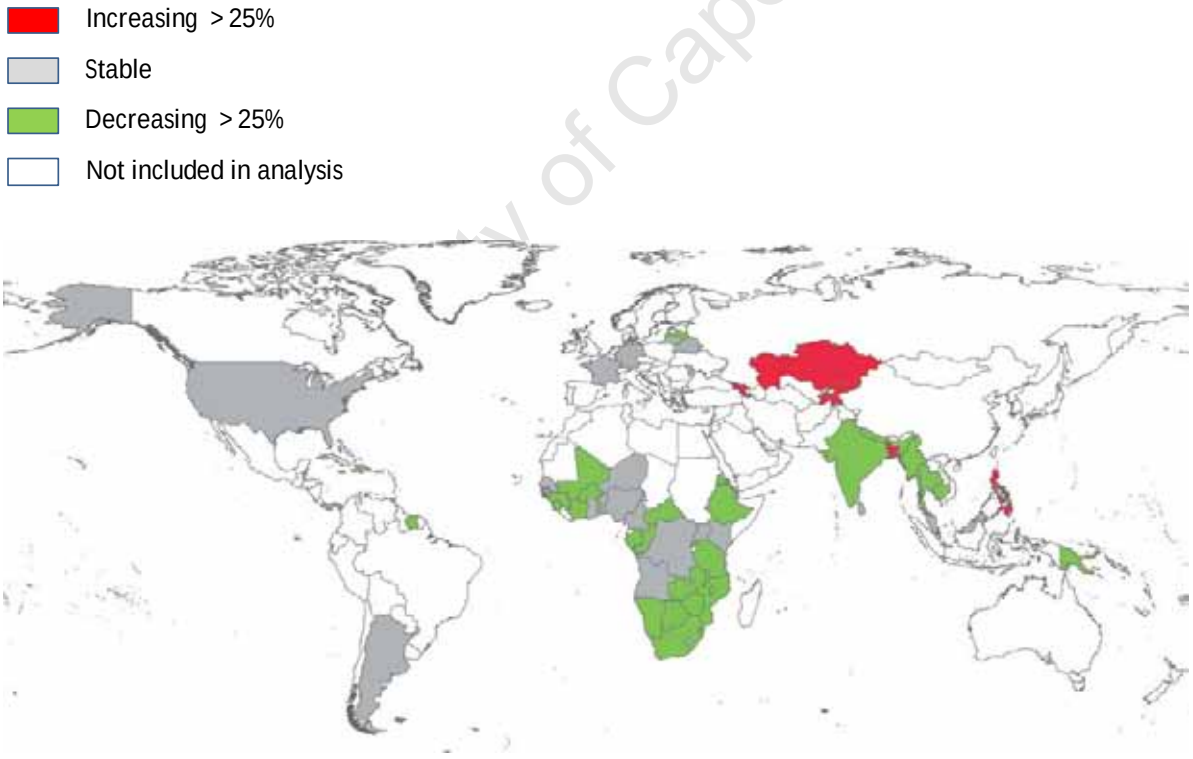

Figure 1.2 Global trends of changes in the incidence rate of HIV infections between 2001 and 2009. The map includes trends from 60 countries where reliable diagnostic tests were used to determine the HIV incidence rate and 3 countries where peer reviewed of publications with incidence trends were available. Incidence rates could not be concluded for countries such as Brazil, China and Russia Federation due to complex epidemics and were omitted from the 2010 estimations (UNAIDS 2010).

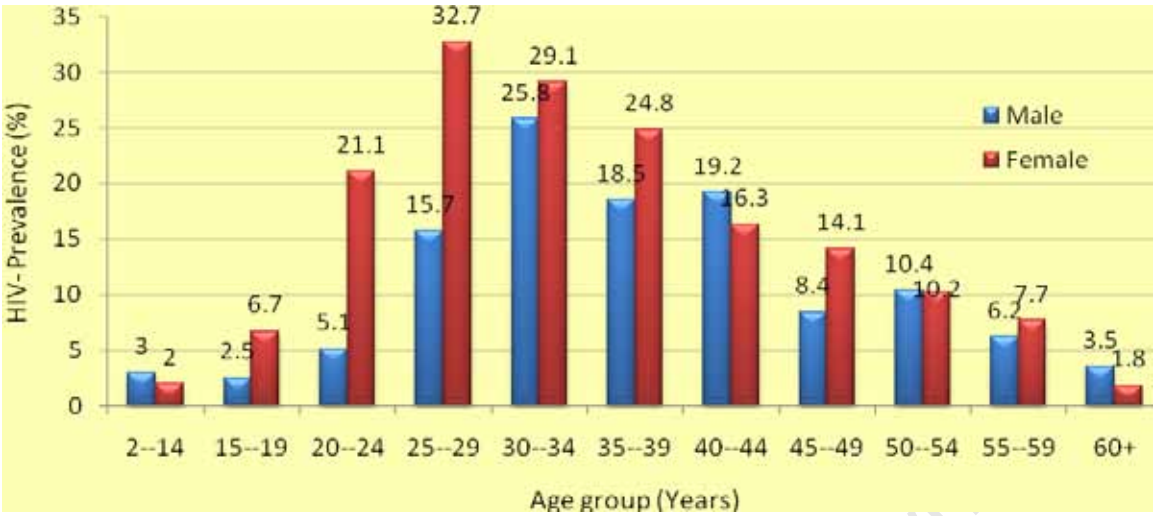

Figure 1.3 The 2008 survey of HIV prevalence by gender and age in South Africa. Generally, women had a higher HIV prevalence than men with the exception of age groups 40-44, 50-44 and the 60+ groups. This may be an indication of intergenerational sexual practice where younger women are easily pressurized in not using condoms (adapted from RSA HIV/AIDS Progress Report, 2010).

1.2 Phylogenetics of HIV

HIV belongs to the Lentivirus genus and is a subfamily member of the Retroviridae group of viruses. HIV-1 is closely related to simian immunodeficiency virus (SIV) from chimpanzees and HIV-2 is closely related to SIV from sooty mangabeys (Hirsch *et al*., 1989; Gao *et al*., 1999). Of these two HIV subtypes, HIV-1 is more prevalent globally whereas HIV-2 is endemic in West Africa and sporadically in the rest of the world. HIV-2 is also thought to be less pathogenic and is associated with a slower rate of disease progression than HIV-1 (reviewed by Rowland-Jones and Whittle, 2007).

HIV-1 has been further divided into three distinct groups: M (majority), N (non-M and non-O) and O (outlier) as shown in Figure 1.4. HIV-1 group M can be further sub-divided into subtypes or clades, namely A, B, C, D, F, G, H, J and K (Figure 1.4). HIV-1 subtype C is the most prevalent clade globally and the most dominant clade found in South Africa and Asia (Hemelaar *et al*., 2006). HIV-1 subtype C accounts for 50% of the total global infection, whereas subtypes A, B, D and G account for 12%, 10%, 3% and 6%, respectively. The least prevalent subtypes F,

H, J and K together account for only 0.9% of infections globally (Hemelaar *et al.*, 2006; Figure 1.5).

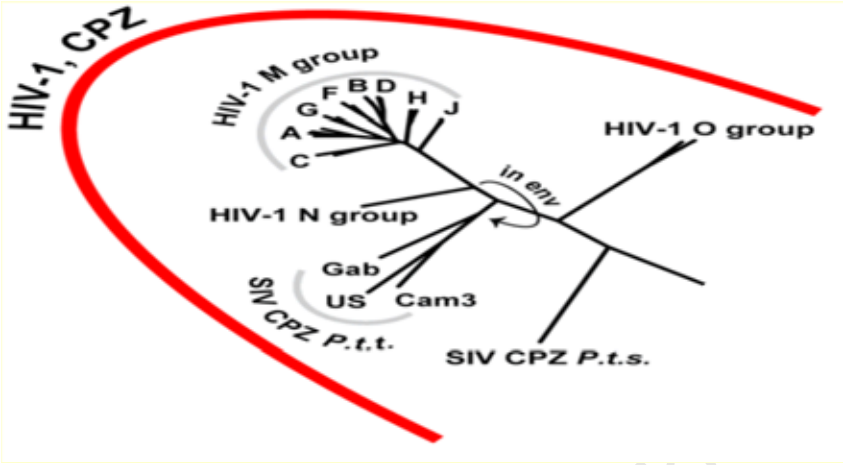

Figure 1.4 Phylogenetic tree of HIV-1 and SIV chimpanzee. The tree shows the three groups of HIV-1 M (main), N (non-M and non-O) and O (Outlier) as well as the close relationship between HIV-1 and SIVcpz. HIV-1 M can be further subdivided into clades: A, B, C, D, F, G and K (not represented in this tree; adapted from HIV Sequence Database).

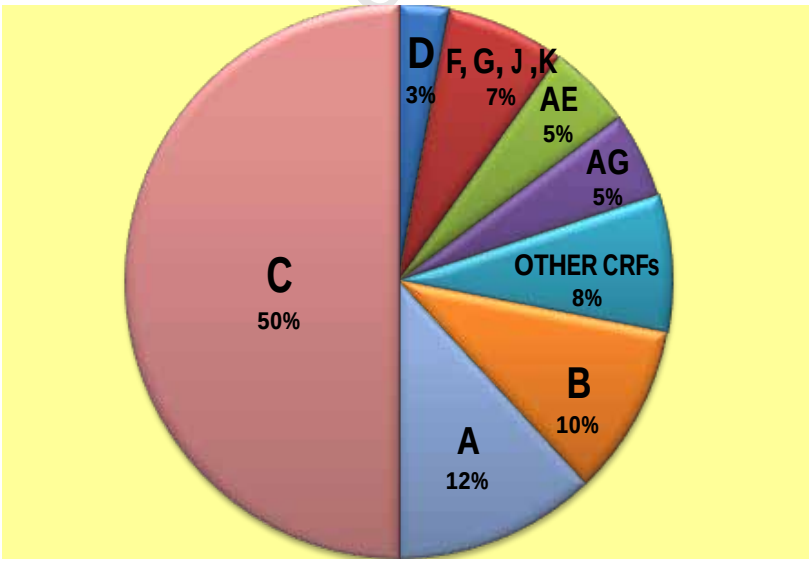

Figure 1.5 Global distribution of HIV-1 subtypes and circulating recombinant forms in 2004. The proportions of subtypes, A, B, C, D, F, G, H, J, K and recombinants AE, AG and other are represented in percentage of the global total number of individuals living with HIV-1 (Hemelaar *et al*., 2006).

1.3 Organization of HIV

HIV is an enveloped retrovirus with an RNA genome of 10 kilobases. The outer envelope of HIV is comprised of a phospholipid bilayer derived from the host cell membrane (Figure 1.6). Protruding from the envelope are spikes, composed of a trimeric complex made up of the hetrodimer glycoproteins gp120 (spike cap) - gp41 (spike stem) (White *et al*., 2010). The inner core of the virus consists of viral enzymes, reverse transcriptase and proteins as well as two identical singular strands of RNA, each of which encodes copies of nine genes. Three of these nine genes (Gag, Pol and Env) encode structural proteins whereas the other six are regulatory genes (Vif, Rev, Nef, Tat, Vpu, and Vpr) and are involved in the control of viral replication. The Gag gene encodes for three structural proteins: p24 (capsid), p17 (matrix) and p15 (nucleocapsid).

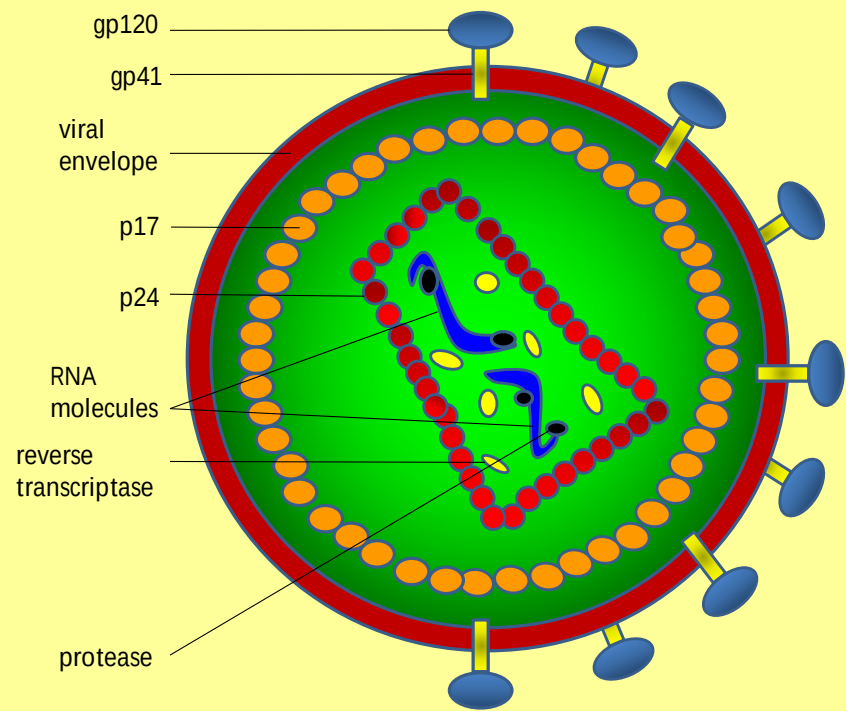

Figure 1.6 Structure of HIV. The outer layer consists of a phospholipid bilayer from which protrudes glycoproteins, gp41 and gp120. The core structure has enzymes, reverse transcriptase and protease as well as two single stranded RNA molecules which encode for the structural proteins (Gag, Pol and Env) and regulatory proteins (Vif, Rev, Nef, Tat, Vpu, and Vpr).

1.4 HIV life cycle in host cells

The HIV-1 replication cycle in host cells is summarized in Figure 1.7. For HIV-1 entry into a target cell, it requires binding of HIV gp120 to host cells expressing CD4; including CD4+ T lymphocytes, monocytes, macrophages and dendritic cells (reviewed by Hladik and McElrath, 2008). Binding of CD4 to gp120 induces a conformational switch in gp120 which facilitates gp120 binding to a chemokine co-receptor on the host cell membrane (Doms and Moore, 2000). Several chemokine co-receptors have been identified but the most common ones used by HIV-1 are either CXCR4 or CCR5 (reviewed by Wu, 2010). Following attachment, the fusion peptide of gp41 allows for the fusion of the viral cell membrane and the host cell membrane (Briggs *et al*., 2000). The process of uncoating of the viral particle occurs next, whereby the viral core is released into the cytoplasm of the target cell, freeing the viral RNA. Viral RNA is converted into proviral DNA by the action of reverse transcriptase and intergrase, and this is followed by the formation of a hybrid RNA/DNA double helix. The RNA is broken down by ribonuclease and polymerase reconstructs the complementary DNA strand to form the double helix DNA molecule, which is transported to the nucleus and incorporated into the host genome by the action of intergrase (reviewed by Fanales-Belasio *et al*., 2010). This double helix DNA is called a provirus and can remain dormant for long periods of time and in this way a host cell can remain latently infected (Zhou *et al*., 2005).

Transcription of proviral DNA into mRNA can only take place once the host cell is in an activated state (reviewed by Fanales-Belasio *et al*., 2010). The initial process of transcription involves the synthesis of regulatory HIV-1 proteins, followed by the later stage of expression of structural proteins. Once new viral particles are formed, they migrate towards the cell membrane where proteins from the host cells such as cholesterol and phospholipids are included and the immature virions start budding (Adamson and Freed, 2007).

Figure 1.7 HIV replication cycle in host cells. To infect a new host cell, HIV first interacts with host cell CD4 using gp120 and gp 41. This interaction causes a conformational change in gp120 which allows interaction with a chemokine co-receptor, leading to fusion and entry of the virion into the host cell. Following entry, viral RNA is copied into DNA by reverse transcriptase which is incorporated into the host DNA. At this stage the integrated DNA is known as a provirus which is transcribed into mRNA. Translation and assembly of new HIV structural proteins follows by using the proteins from the host to form virions which bud from the cell membrane.

1.5 The natural history of HIV infection

Once HIV has infected a new human host, the clinical disease course that follows HIV-1 infection can be divided into three distinct clinical stages: the acute phase of HIV infection, the chronic phase of HIV infection in the absence of anti-retroviral therapy, and finally acquired immune deficiency syndrome (AIDS) associated with a decline in CD4+ cells to <200 cell/µl in blood (Figure 1.8). The acute phase is the focal point of this study.

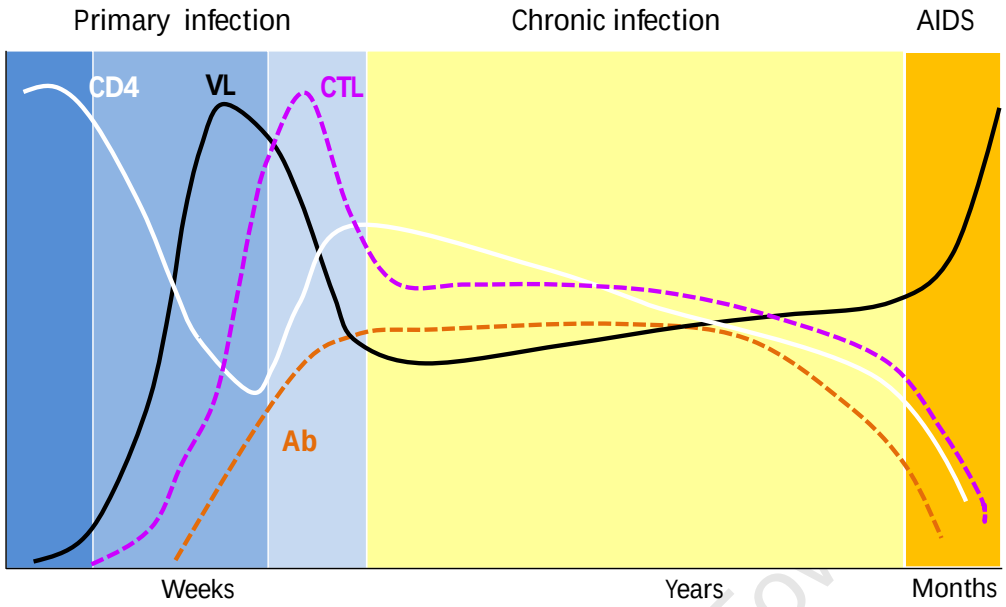

Figure 1.8 Kinetics of HIV disease progression in the absence of anti-retroviral treatment. The initial extraordinary rise in HIV load in the acute phase is followed by an increase in HIV-specific CTLs and a decrease in the number of $CD4^+$ T cells. HIV RNA load is reduced within 1-2 months and maintained at a new lower threshold by the immune response. The lower HIV RNA load is concomitant with an increase in $CD4^+$ T cell numbers, and at 2 to 3 months seroconversion occurs as HIV-specific antibodies appear. This new symptomatic phase is predominantly maintained for a number of years. A slow decrease in the $CD4^+$ T cell counts leads to the onset of AIDS elevating susceptibility to other pathogenic infections. This is followed by an increase in the virus load, along with a decrease in the HIV-specific CTL, and neutralizing antibodies.

The acute phase of HIV infection represents the first interaction between the human immune system and the incoming virus (Johnston and Fauci, 2008; Yang *et al.*, 2008; Boutwell *et al.*, 2010). HIV-specific T cells are detected within 4-6 weeks of infection while HIV-specific binding antibodies (associated with HIV seroconversion) generally appear within 1-3 months post-infection (Kahn and Walker, 1998). The emergence of HIV-specific T cells is associated with a marked decline in plasma viremia which eventually stabilizes between 6 and 12 months of infection to a level known as viral set point (Boutwell *et al.*, 2010). HIV set point is the level to which the host immune response can control plasma viremia: the lower the viral set point, the better prognosis for the long term survival of an infected individual (Mellors *et al.*, 2007; Streeck *et al.*, 2009).

The chronic or asymptomatic phase follows and can last for many years, where there is constant viral replication with gradual but irreversible loss of CD4 T cells (Centlivre *et al*., 2007). In the absence of anti-retroviral therapy, the final stage, AIDS, occurs when the majority of CD4 T cells have been destroyed and the immune system can no longer mount effective responses against opportunistic pathogens (Centlivre *et al*., 2007).

1.5.1. Clinical course of acute HIV infection

Acute HIV infection refers to the period immediately after productive infection where HIV RNA is detectable in blood but HIV-specific antibodies may not yet be detectable. Acute HIV-1 infection is characterized by a dramatic increase in titers of plasma HIV and significant loss of CD4 T cells (Khan *et al.,* 1998), particularly in the gut-associated lymphoid tissue (Mattapallil *et al.,* 2005). Although transient, clinical symptoms associated with acute HIV infection do occur shortly after exposure (Machala *et al.,* 2004), including fever, rash, headaches, myalgia lymphadenopathy, pharangitis, arthalgia, gastrointestinal distress, night sweats, and oral or genital ulcers (Schacker *et al.,* 1997). The time from infection to the onset of first clinical symptoms is usually 2-6 weeks, with seroconversion starting 1-3 weeks later (Petersen *et al.,* 1994).

Fiebig *et al.* (2003) has defined six clinical stages of acute HIV infection based on the presence or absence of HIV-1 specific antigens or antibodies in the blood of an HIV-infected individual (Figure 1.9). Stages I–VI are determined by the sequential gain of positive reactions to viral RNA (PCR), p24 and p31 (ELISA) and viral antibodies (western blot). Prior to the Fiebig stage I, is the eclipse phase, which lasts for 0-10 days from viral exposure (Khan and Walker 1988; McMichael *et al*., 2010). During this phase, there is no detectable viral RNA in the blood. Following the eclipse phase, viral RNA levels rise exponentially in plasma, peaking at about 28-30 days (Fiebig stages I-IV) and then subsequently declining (Fiebig stage V) until a plateau is reached during early chronic infection (Fiebig stage VI).

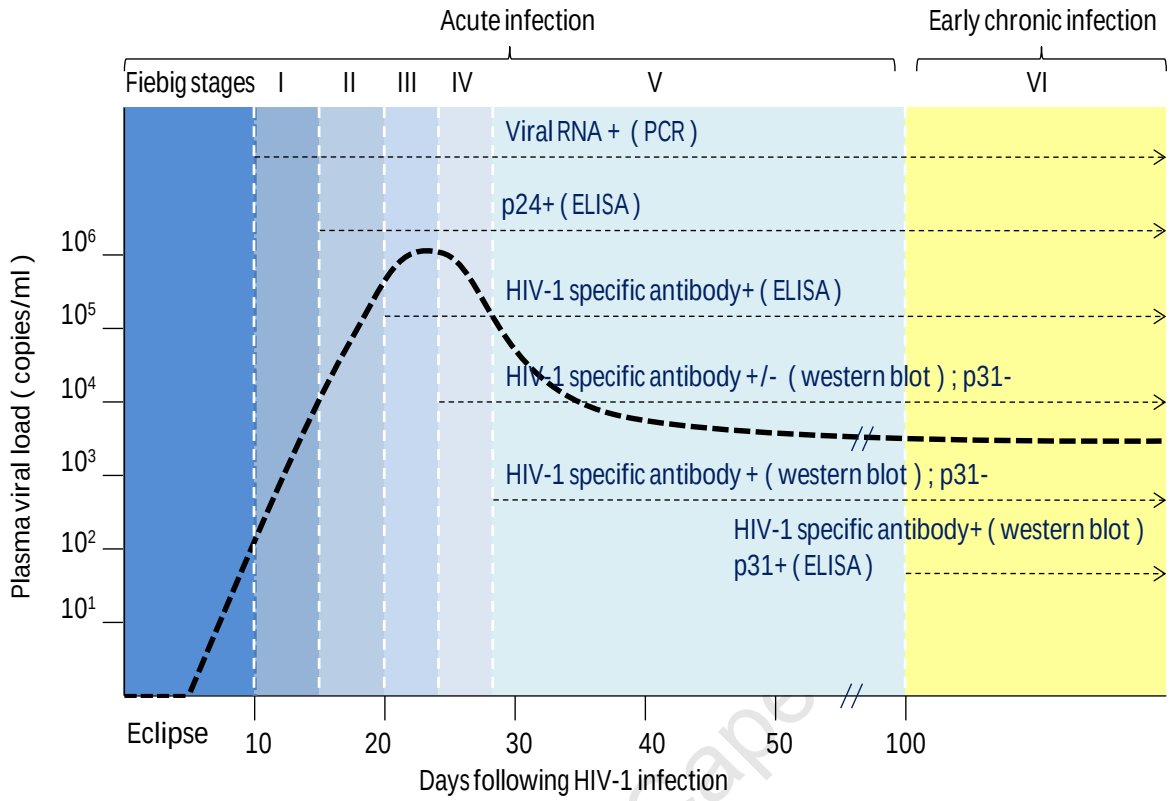

Figure 1.9 Fiebig staging of acute HIV infection. Fiebig *et al*. (2003) defined a six-stage (I-VI) classification system for acute HIV infection using the stepwise gain in positivity for the detection of HIV-1 specific antigens and antibodies. The period between 0-10 days is known as the eclipse phase. This is followed by an exponential increase in plasma viral RNA levels which peak between 21 and 28 days post-infection (Fiebig stages I – III). After the initial burst in viremia, a slower decrease in viral RNA follows at approximately 100 days post infection (Fiebig stages IV – V). Fiebig Stage VI is the start of the early chronic phase and this usually occurs when the viral load begins to plateau (McMichael *et al*., 2010).

1.5.2. CD4 T cell counts and viral load fluctuations during acute infection

CD4 T cell counts in blood and HIV plasma load measurements are independently strong predictors of HIV disease progression, however the combination of the two can strengthen the monitoring and evaluation of patients infected with HIV during all stages of disease (Mellors *et al*., 1997). It is well documented that the initial burst of viremia reaches a peak of approximately 10^5-10^7 RNA copies/ ml with a simultaneous profound loss of $CD4^+$ T cells (Daar *et al*., 1991; Clark *et al*., 1991). The fast decline of $CD4^+$ T cells is followed by a slower rate after 5 to 6 months of infection. During acute HIV-1 subtype C infection, recent studies have shown that

plasma viral load was shown to be inversely associated with $CD4^+$ T counts (Gray *et al*., 2005; Novitsky *et al.*, 2009). Furthermore, during acute HIV-1subtype C infection, Novitsky *et al.* (2009) found that individuals whose peak viral loads declined fast had lower viral load at set point and better preservation of CD4 T cell numbers during the course of infection than individuals whose viral loads declined more slowly.

1.5.3. CD4 T cell counts and viral load fluctuations during chronic infection

Approximately six months after infection, most individuals reach an asymptomatic phase with a viral set point often lower than 20,000 RNA copies per ml (reviewd by Levy, 2009). However the viral set point value may vary up to a 1000 fold between patients (Mellors *et al*., 1996; de Wolf *et al*., 1997). Following ten years of infection, about 50% of untreated HIV-infected individuals will drop their CD4 T cell counts below 350 cells per µl and start exhibiting signs of other opportunistic infections (progressors; Buchbinder *et al*., 1994 and reviewed by Levy, 2009). A small percentage of individuals (~5%) are able to maintain CD4 T cell counts above 500 cells per ml for several years and are know as long term non-progressors (LTNPs). Less than 1% of these individuals are able to maintain low viral load of <50 copies/ml for many years and have been defined as elite controllers (ECs). Most of these ECs exhibit very low rates of CD4 decline (reviewed by Blankson, 2010). Moreover, elite controllers characteristically have a slower rate of disease progression than LTNPs who inevitably develop progressive HIV-1 (Rodés *et al*., 2004; Blankson, 2010).

Several studies have suggested that LTNPs and ECs may be infected with a defective virus (Alexander *et al*., 2002; Calugi *et al*., 2006), which partially explains their ability to control their viraemia. However, others have shown that, in some instances, these individuals could be infected with a pathogenic virus. Miura *et al*. (2008) showed by viral sequencing that there were no large deletions present in 60 ECs which reveal that these virus are replication-competent. A subsequent study showed that a pathogenic virus from a progressor who developed AIDS was transmitted to an EC (Bailey *et al*., 2008; reviewed by Blankson 2010).

Similar to acute infection, there is evidence of inverse correlation between CD4 T cell count and viral load during chronic untreated infection (Mendiratta *et al*., 2009). In a recent review, it was shown that 7% of individuals from two controller cohorts infected for longer than 16 years with

viral load less than 75 RNA copies per ml had met the clinical definition of AIDS, with CD4 T cell counts less than 350µl per ml. This suggests that ECs with low viral loads in the absence of therapy are not always able to maintain their CD4 T cell counts during the course of infection (Poropatich and Sullivan 2011).

1.6 HIV-1 specific immune responses

Humoral and cellular immune responses have been implicated in control of HIV infection and HIV-specific antibody and T cell immune responses are readily detected in HIV-infected individuals (Figure 1.8; Fiebig *et al.*, 2003). HIV-specific CD8 T cell responses during acute infection emerge as early as two weeks post-infection while neutralizing antibodies are only detectable several months later (Figure 1.8; Pilgrim *et al.*, 1997; Gray *et al.*, 2007). Whereas antibodies generally recognize unprocessed conformational or linear epitopes of HIV, T cell immunity against HIV requires intracellular processing of HIV in antigen presenting cells and presentation of short HIV linear peptides bound to Major Histocompatibiliy Complex (MHC) or human leukocyte antigen (HLA) molecules on the surface of antigen presenting cells (Zinkernagel and Doherty, 1997).

1.6.1. Role of HIV-specific antibody responses in the control of HIV-1 infection

HIV-specific binding antibodies are generally detectable within weeks of infection and are the basis of the Rapid test used to diagnose infection with HIV (HIV seroconversion; Figure 1.8; Pilgrim *et al.*, 1997; Gray *et al.*, 2007). HIV-specific antibodies capable of neutralizing the virus are generally only detectable months after the first binding antibodies are detected. This lag in the development of neutralizing antibodies has also been reported for SIV in macaques (Reimann *et al.*, 1994; Montefiori *et al.*, 1996). No correlation was found between the emergence of HIV-specific neutralizing antibodies following infection and decline in viremia during acute infection, even though binding Env-specific antibodies were detectable as early as two weeks after onset of symptoms (Aasa-Chapman *et al.*, 2004; Gray *et al.*, 2007). This has lead many to speculate that neutralizing antibodies against HIV are not important in the initial control of HIV infection. It is, however, likely that anti-HIV antibodies would be protection against infection if they were present prior to infection as a consequence of an effective HIV vaccine.

Some studies have shown that neutralizing antibodies develop more frequently in LTNPs than progressors (Cecilia *et al*., 1999) while others show that they are not (Pereyra *et al*., 2008; Doria-Rose *et al*., 2010). Pereyra *et al*. (2008) reported that ECs had lower levels of heterologous neutralizing antibodies than individuals with higher viral loads. Furthermore, Blankson *et al*. (2010) showed that individuals with very low levels of virus (using very sensitive assays) had the lowest concentration of neutralizing antibodies suggesting that the neutralizing antibodies did not contribute to the maintenance of low viral load.

1.6.2. Immune control by CTLs during acute HIV-1 infection.

In contrast to antibodies, there is a substantial amount of evidence to suggest that CD8+ cytotoxic T lymphocytes (CTLs) are able to control HIV replication during acute infection. Koup *et al*. (1994) and Borrow *et al*. (1994) showed that the eradication of acute viral symptoms and concomitant reduction in viral load was temporarily associated with the emergence of HIV-specific cellular immunity. Subsequently, SIV-infected macaques in which CD8+ T cells were depleted prior to infection by CD8+ monoclonal infusion had higher viremia and accelerated disease course than immuno-competent macaques (Jin *et al*., 1999; Schmitz *et al.*, 1999). HIV-1 specific cellular immune responses generated during early HIV infection appear to be key determinants of the rate of disease progression (Mellors *et al.*, 1996; Allen *et al*., 2000; Streeck *et al.*, 2009; Streeck and Nixon 2010).

1.6.3. Characterization of HIV-1 specific immune responses during acute and chronic HIV infection

While there is strong evidence that specific CTL responses are important in HIV control, a number of studies have reported inconsistent associations between magnitude and/or breadth of HIV-specific CTL responses and viral load. Some studies reported an inverse correlation between HIV specific T cell responses with plasma viral load (Ogg *et al*., 1998; Betts *et al*., 1999; Buseyne *et al*., 2002; Chouquet *et al*., 2002; Edwards *et al*., 2002) where others observed a positive correlation (Masemola *et al*., 2004). In contrast, Addo *et al*. (2003) and Novitsky *et al*. (2003) have shown no relationship between HIV specific T cell responses and plasma viral load. With the use of 410 overlapping peptides spanning the entire HIV-1 consensus B genome including multiple ethnic groups, Frahm *et al*. (2004) found that Gag- and Nef-specific responses were detected in all groups but that there was no correlation between the magnitude and breadth

of T cell responses and viral load. Masemola *et al.* (2004) in subtype C infection (mixture of early and chronic cohort) and Lichterfeld *et al.* (2004) in subtype B infection (primary cohort) showed that Nef was the most frequently targeted of all HIV proteins although both studies found no correlation between viral load and the breadth of HIV-specific responses and only a weak correlation between viral load and the magnitude of responses. In more recent longitudinal studies of acute and early infection, it was confirmed that Nef-specific responses were immuno-dominant (Turnbull *et al*., 2009; Mlotshwa *et al*., 2010)

It was also observed that early HIV-specific T cell responses during acute infection were lower in magnitude and directed against only a small number of epitopes than those detected during the chronic infection which were also broader (Dalod *et al*., 1999; Altfeld *et al.,* 2001; Yu *et al.,* 2002; Addo *et al*., 2002; Turnbull *et al*., 2009). In contrast, in a longitudinal study during chronic infection, the sequence evolution of CD8 T cell epitopes remained relatively constant over time, despite the continued persistence of CD8 T cell response (Koibuchi *et al*., 2005).

Several of the abovementioned studies employed the IFN-γ ELISPOT assay which generally does not distinguish between CD4 and CD8 T cell responses unless purified CD8+ or CD4+ T cell populations are used in an experiment. In a study where CD8 cells were depleted, however, no correlation was found between the magnitude or breadth of HIV-specific CD4+ T cell responses and viremia (Lichterfeld *et al.,* 2004). Betts *et al*. (2001) reported a similar finding for CD4+ T cells using intracellular cytokine staining (ICS) and flow cytometry but showed a positive correlation between the total frequency of HIV-specific CD8+ responses and viral load.

Only a few recent studies have characterized the kinetics of HIV-specific T cell immune responses longitudinally following acute phase of infection (Turnbull *et al*., 2009; Goonetilleke *et al*., 2009; Mlotshwa *et al*., 2010). All three studies showed that specific T cell responses were not static but expanded and contracted in subsequent waves resulting in shift in the epitope immunodominance (Turnbull *et al*., 2009) and temporal patterns due to viral sequence variance and recognition invariant viral epitopes (Mlotshwa *et al*., 2010). In addition CD8+ T cells–mediated killing of HIV-infected target cells were involved much earlier during acute infection than initially speculated (Goonetilleke *et al.,* 2009).

1.7 Immune escape from HIV-specific CTLs during acute and chronic HIV infection

Although CTLs responses play an important role in the initial control of HIV replication and disease progression, their ability to control HIV replication is impacted by the appearance of viral escape mutations (Moore *et al*., 2002). CTLs recognize 8-11 amino acid long peptides presented by HLA class I molecules (reviewed by Yewdell *et al*., 2003). Because CD8+ T cell immunity is dependent on recognition of antigenic peptides bound to specific HLA class I molecules, the "fit" of these peptides into HLA binding grooves directly influences the ability of a particular T cell to respond to its cognate antigen. Any alteration or mutation within the foreign peptide or the antigenic binding cleft of the MHC molecule can alter T cell recognition and this is a mechanism used by some viruses to evade T cell immunity (Moore *et al.*, 2002). The loss of viremic control during HIV infection is often associated with changes in the viral sequence targeted by effective CTL responses which results in the impairment of viral recognition by CTL or alteration in antigen presentation (Jones *et al*., 2004; Draenert *et al.*, 2004; Martinez - Picado *et al*., 2006).

A study by O'Connor *et al.* (2002) in non-human primates showed that SIV is able to escape from the immune pressure being exerted by CTLs very early during infection by mutating the viral epitopes being targeted by the CTLs. Many studies have since confirmed that this was a common feature of acute (Borrow *et al*., 1997; Cao *et al*., 2003; Allen *et al*., 2004; Bernardin *et al*., 2005; Li *et al.*, 2007; Brumme *et al.*, 2008; Wang *et al*., 2009) and chronic (Draenert *et al.*, 2004; Goepfert *et al*., 2008) HIV infection in humans.

Certain HLAs have been associated with better HIV disease prognosis while others are associated with poor prognosis. For example, HLA-B*27 and –B*5701, B*5703, B*5801 and B*-51 are associated with slow disease progression whereas HLA-B*35 and B*5802 are associated with rapid onset of AIDS (Gao *et al*., 2001; Carrington and O'Brien, 2003; Goulder and Watkins, 2008). HIV epitopes that are restricted by the protective HLAs have been shown to be more frequently recognized than those that are restricted by the non-protective HLAs, irrespective of the stage of disease (Altfeld *et al.*, 2006). Furthermore, Frater *et al*. (2007) showed that individuals with HIV epitopes restricted by beneficial HLAs had more mutations than individuals lacking these beneficial HLAs. This suggests that the epitopes that were

restricted by the beneficial HLA were more frequently recognized which leads to higher selection pressure by CTL, which is evidenced by the higher rate of mutations.

1.8 Impact of immune escape on HIV fitness

Although there are numerous studies demonstrating that HIV is capable of mutating to escape CTL detection during both acute and chronic HIV infection (Goulder *et al*., 2004), each immune escape may result in some fitness cost to the virus (Li *et al.*, 2007). When a mutated virus is transmitted to a new host with different HLA alleles to the donor, epitopes that are no longer restricted by the HLA of the new host can revert to its original ancestral or wild type form at no fitness cost to the virus (Leslie *et al.*, 2004; Crawford *et al.*, 2007; Li *et al.*, 2007). When mutations do not revert after transmission in these hosts, however, this suggests that the mutation was not significant (Leslie *et al*., 2004; Draenert *et al*., 2004) or that viral fitness was restored by compensatory mutations (Kelleher *et al*., 2001; Schneidewind *et al*., 2007). Futhermore, Chopera *et al.* (2008) showed that HLA mismatched individuals who have received transmitted viruses which have known attenuating mutations (T242N and A146X) as a result of coming from an HLA B*57/B*5801 donor (associated with LTNPs) exhibited effective control of vireamia following HIV infection.

Certain parts of the HIV genome are highly conserved (such as the structural proteins of Gag; Edwards *et al*., 2002) but there are also regions that are highly variable and easy for the virus to mutate without significant fitness costs (such as envelope; Kiepiela *et al*., 2007). Studies have shown that CTLs targeting conserved region of HIV are beneficial to the host because the virus is less likely to escape these CTL responses due to high fitness costs associated with escape in these regions (Martinez–Picado *et al*., 2006; Goepfert *et al*., 2008).

1.9 Immunodominance in HIV-1 specific CTL responses

Yu *et al*. (2002) described CTL responses directed against HLA-A*3 and HLA-B*7 restricted epitopes following acute infection in an individual that were conserved over a period of 34 months post-infection. In the same study, all of the seventeen additional individuals who were

HLA-A*3+ and HLA-B*7+ also had CTLs targeting one or both of these HLA-A*3 and HLA-B*7 epitopes during acute infection, suggesting that patterns in the development of immunodominant CTL responses exist.

Streeck *et al.* (2009) investigated the relationship between the first immunodominant specific T cell responses during acute infection with those in chronic infection. They showed that CTL responses developed in a hierarchical immunodominant pattern during acute infection and correlated with viral set point, and a slower rate of CD4 decline. In contrast, these authors did not find a correlation between immundominant CTL responses during chronic infection and viral set point.

Individuals who mount an immunodominant HIV-specific response at the onset of HIV-infection have been shown to have a greater chance of a having a lower viral set point than those who do not target immunodominant epitope during acute infection (Goonetilleke *et al*., 2009). It is also possible to predict which HIV epitopes are likely to be targeted during HIV infection in individuals who have known HLA types (Streeck and Nixon, 2010). For example, an individual who is HLA-B*27+ has an 81% chance of targeting the Gag KRWIILGLNK [KK10] epitope during acute infection and this drops to a 43% chance during chronic infection. An individual who is HLA-A*30+ has a 25% chance of targeting Integrase KIQNFRVYY [KYY9] epitope during acute infection and an 80% chance of targeting this epitope during chronic infection.

1.10 Importance of HIV-specific CTLs targeting Gag

Early studies have shown that Gag-specific CTLs are associated with better viral control than CTL responses directed against other HIV proteins (Edwards *et al.*, 2002; Novitsky *et al*., 2002; Buseyene *et al.*, 2002; Masemola *et al*., 2004 and Ramduth *et al*., 2005). These studies have suggested that this better control is because Gag is a highly conserved structural protein that does not tolerate escape mutations easily and in which mutations are often accompanied by substantial fitness cost to the virus. In addition, and possibly as a result of it being so conserved, Gag also has high CTL epitope density.

Kaushik *et al*. (2005) also showed that p24 was the most recognized region within Gag while Mendiratta *et al*. (2009) showed that the magnitude of Gag-specific responses correlated inversely with viral load whereas Nef-specific responses were not correlated with viral load. In a large South African cohort of 578 untreated chronically HIV-infected participants, Kiepiela and colleagues (2007) showed that individuals who targeted Gag had significantly lower viremia than individuals who did not whereas individuals who targeted Env had significantly higher viremia than those that did not (Kiepiela *et al*., 2007). This confirms that Gag-specific T cell responses are associated with effective control of viremia.

HLA-B*57, one of the most protective HLA alleles, has been shown to restrict epitopes within the p24 region of Gag: **T**STLQEQIA**W** [TW10], **K**AFSPEVIPM**F** [KF11] and **I**SPRTLNA**W** [IW9] (Goulder and Watkins, 2008). TW10 is the most frequently targeted B57 restricted epitope during early infection and was also found to escape CTL pressure the most rapidly (Streeck *et al*., 2007; Brumme *et al*., 2008). TW10 can also be presented by HLA B*5801, which is closely related to HLA B57 (Goulder *et al*., 1996). The most common mutation within this B*57/B*5801 restricted epitope is T242N, which occurs rapidly during acute infection (Brumme *et al.*, 2008) and is associated with a replication-incompetent virus (Martinez-Picado *et al*., 2006; Miura *et al.*, 2009). Individuals with T cell responses targeting the HLA B*27 epitope in Gag **K**RWIILGLN**K** (KK10) were also shown to control viremia whereas individuals who expressed the same HLA but failed to target KK10 typically had high viremia (Goulder and Watkins, 2008). These experiments show the strong impact of HLA-B phenotype and the targeting of Gag has in the control of HIV disease progression.

CONCLUSION

Acute HIV infection is associated with the emergence of HIV-specific CTL responses within the first few weeks after infection that are associated with the drop in peak viraemia. Studies have shown that targeting of HIV Gag responses early during HIV infection is associated with better control of viral load. Studying HIV-specific T cell response kinetics during the course of HIV infection may give important insight into the type of CTL specificities associated with better control. This information is likely to be important in the design of an effective HIV vaccine.

RATIONALE OF THIS STUDY

The need to develop an efficacious HIV vaccine to either protect from or attenuate HIV-associated disease progression is paramount. Better understanding of the correlates of protective T cell immunity associated with control during HIV infection is central to effective vaccine design. Previous studies have suggested that immune responses mounted early during HIV-infection determine the level of control of viremia (HIV set point) achieved in an individual (Mellors *et al.*, 1996; Allen *et al*., 2000; Rosenberg *et al*., 2000; Streeck *et al.*, 2008).

The correlation between HIV set point and the rate of disease progression (Streeck *et al*., 2009) further suggests that HIV-specific immune responses generated during acute/early infection are important to understand. In this study, the magnitude and breadth of cellular immune responses against HIV Gag and Nef were monitored longitudinally in acutely HIV infected individuals through to their chronic stages of infection by IFN-γ ELISPOT.

AIM

To investigate the relationship between HIV disease progression and both the magnitude and persistence of HIV-1 subtype C Gag and Nef-specific T cell responses during acute through to chronic HIV infection.

Specific Objective 1

To compare the magnitude and breadth of HIV-1 Gag- and Nef-specific T cell responses generated during acute HIV infection with those from chronic HIV infection.

Hypothesis: *HIV-specific immune responses during acute infection will be narrower than responses mounted during chronic infection but will broaden with increasing disease progression. HIV-specific immune responses in long-term non-progressors will be higher in magnitude and remain more stable over time than those detected in individuals during acute infection.*

Specific Objective 2

To investigate the relationship between clinical markers of HIV disease progression (plasma HIV RNA load and blood CD4 T cell counts) and the magnitude and/or breadth of HIV-specific responses in individuals who are acutely infected with HIV compared to long-term non progressors.

Hypothesis: *Individuals with the highest magnitude and breadth of responses to HIV Gag and Nef in acute infection and to Gag only in LTNPs will show better ability to control HIV disease progression compared to individuals with low magnitude narrow responses.*

CHAPTER 2

Material and Methods

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2.1 Description of cohort

Five hundred HIV negative individuals from Gugulethu, Cape Town were enrolled in an observational vaccine preparedness study headed by Dr Linda-Gail Bekker from the Desmond Tutu HIV Foundation and funded by the International AIDS Vaccine Initiative (IAVI). University of Cape Town Ethics approval for the study was obtained and all participants gave informed written consent to participate in the study and offered voluntary risk reduction counselling and HIV testing monthly for 12 months. Each participant was screened for HIV antibodies and RNA at each monthly visit to determine the HIV incidence rate in this community. For the purposes of this study, recent HIV infection was defined by at least two positive rapid HIV antibody tests and/or a positive HIV RNA test. The last documented negative HIV antibody test had to have been within 3 months of enrolment.

Of these 500 HIV negative participants, 7 became infected with HIV during follow-up and the HIV incidence was calculated to be 1.4%. Upon HIV infection, individuals were enrolled into an acute HIV-infection cohort and followed up at enrolment, 1 month, 3 months, 6 months, 12 months, 18 months, 24 months and 30 months post-infection. In this study, enrolment was considered acute infection, 1- 3 months were considered to be early infection; while 6-30 months were considered to be chronic HIV-infection.

In addition, 9 individuals who were considered to be long-term non-progressors (who had been infected with HIV for >5 years and maintained CD4 counts >300 cell/ µl in the absence of therapy) were enrolled from the Nyanga Day Hospital in Cape Town for comparison. All participants from the acute infection and long-term non-progressor (LTNP) cohorts were not on highly active anti-retroviral therapy (HAART) at the time of study and clinical data was available from both cohorts.

2.2 Sample collection

At each visit, 80 ml whole anti-coagulated blood was collected by venipuncture into Acid Citrate Dextrose (ACD) vacutainer tubes (BD Bioscience) from each of the study participants by the study nurse. All blood samples were processed immediately upon arrival in the laboratory. Fresh peripheral blood mononuclear cells (PBMCs) were used for assays from the acute study while cryo-preserved PBMCs were used for assays from the LTNP study.

An additional 5ml EDTA (Ethylenediaminetetraacetic acid**;** BD Bioscience) sample was collected from each participant for CD4 counts.

2.3 Sample handling

All laboratory procedures were done in a Biohazard Class II safety cabinet. Protective gowns, gloves and eyewear were worn when handling samples. All waste was placed in disinfectant before it was autoclaved and appropriately disposed of. Regular quality control was done on all instruments and Good Laboratory Practice was strictly adhered to at all times.

2.4 Blood CD4 counts and plasma viral load determination

CD4 counts were performed on all individuals at each visit and done by the National Health Laboratory Services at Groote Schuur Hospital and Green Point, Cape Town. Viral loads were determined in plasma samples from acutely and LTNP HIV-infected women using the NucliSENS EasyQ HIV 1 Version1.2 assay and performed by the National Health Laboratory Services at Groote Schuur Hospital. This assay is reported to have a detection limit of >50 HIV RNA copies/ml. Plasma was derived from ACD anti-coagulated whole blood following Ficoll density gradient centrifugation as previously reported (Gumbi *et al.,* 2008) and stored at -80^{o}C until analysis.

2.5 Isolation of peripheral blood mononuclear cells

PBMCs were isolated from whole blood by Ficoll gradient centrifugation using Leucosep tubes (Greiner Bio-One). Leucosep tubes contain circular porous discs which stabilize the gradient interface and allow for easy harvesting of PBMC without red blood cells contamination. Initially, Histopaque (Sigma-Aldrich) and wash buffer (1% Foetal Bovine Serum in Phosphate buffered saline; Gibco) were equilibrated to room temperature. Histopaque (15 ml) was aliquoted onto the barrier of the 50ml Leucosep tubes. Leucosep tubes were then centrifuged (Heraeus 1.0R Megafuge) at 1257g for 1 min, which allowed the Histopaque to move through the barrier to the bottom of the tube. Histopaque is a viscous density gradient medium consisting of a mixture of polysucrose and sodium diatrizoate solution (Bøyum, 1968). The principle of this method is based on differential densities of mononuclear cells, erythrocytes and granulocytes. When whole blood is layered onto

Histopaque and a centrifugal force is applied, lighter mononuclear cells are held at the plasma interface while heavier erythrocytes and granulocytes gravitate to the bottom of the gradient (Bøyum, 1968).

In this study, well mixed anti-coagulated blood was carefully poured onto the Leucosep barrier and the tubes were centrifuged for 15 minutes at 1257g (Heraeus 1.0R Megafuge). Figure 2.1 outlines the principle of this procedure and describes the position of the PBMC layer following centrifugation. The PBMC layer (Figure 2.1) was harvested using a plastic sterile disposable pasteur pipette and transferred to a new sterile 50ml tube (Greiner Bio-one) for washing. The wash tube was topped up with wash buffer (1% Foetal Bovine Serum in Phosphate buffered saline) and centrifuged 320 x g (1200rpm). The pelleted cells were resuspended in R10 [10% Foetal Bovine Serum in RPMI 50U penicillin, 50mg/ml streptomycin, 50mg/ml glutamine and 0.8mg/ml Fungin] and counted using a Guava automated counter (Guava Technologies).

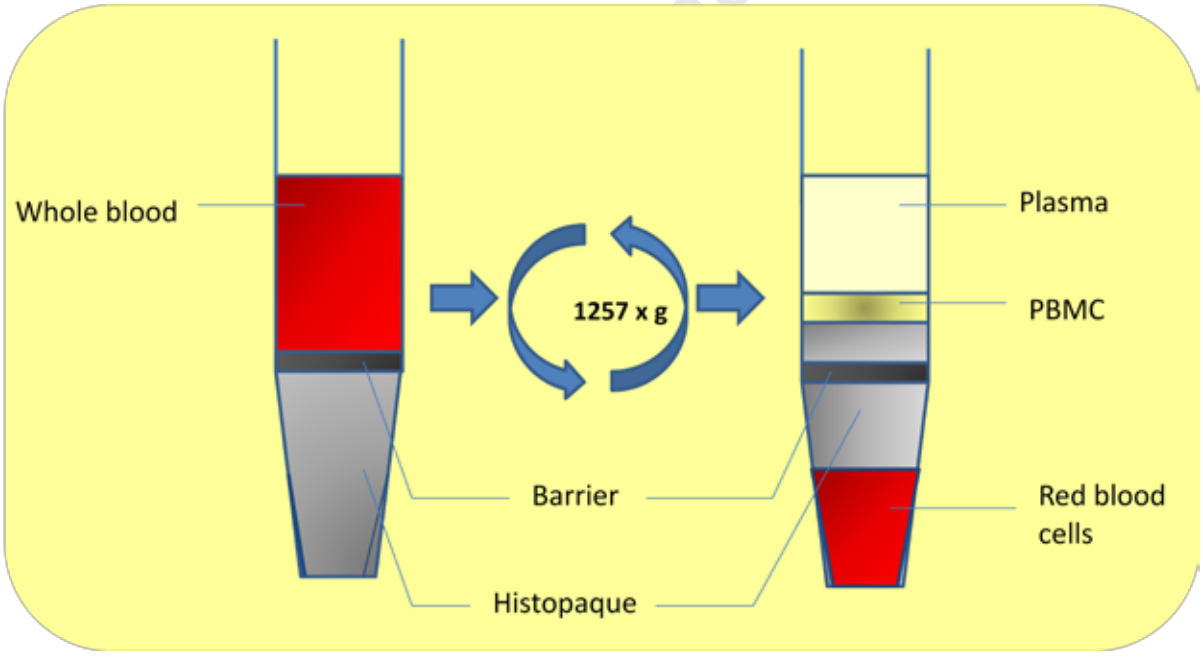


Figure 2.1 Whole blood separation by Histopaque density gradient centrifugation. The tube on the left show layering of mixed whole blood onto the leucosep barrier which is underplayed by Histopaque. The tube on the right shows the result of centrifugation at 1257g for 15 mins. After centrifugation, whole blood has separated into a plasma phase (top), then the layer of PBMCs (also known as the buffy layer), while the heavier red blood cells and granulocytes migrate to the bottom of the gradient.

2.6 Counting of PBMCs

To count PBMCs, 10µl PBMCs were diluted with 190 µl ViaCount reagent (1:20 dilution; Guava Technologies) and incubated at room temperature for 8 minutes. Viacount reagent contains a combination of two DNA-binding dyes with differential permeabilities allowing the distinction between viable and non viable cells. The stained cell suspension was vortexed before counting. A Guava automated cell counter (Gauva Technologies) was used to determine the number of PBMCs isolated. A minimum of 1000 cells were acquired and Cytosoft 2.1.4 software was used to determine the cell concentration. For ELISPOT, PBMCs were adjusted to $2x10^6$ cells/ ml. All remaining PBMCs were frozen in liquid nitrogen for later experiments.

2.7 Cryopreservation of PBMC

Cryopreservation allows long term storage of cells in liquid nitrogen with minimal damage to the cells upon recovery. Dimethyl Sulfoxide (DMSO; Sigma Aldrich) is traditionally used as a cryo-protectant which reduces the formation of ice crystals during the freezing process thus lowering the ionic stress of the cells (Maecker *et al.,* 2005).

PBMCs which were not used immediately for ELISPOT were suspended in a specific volume of neat Foetal Bovine Serum at a final concentration of $20x10^6$ cells/ ml. An equal volume of cold freezing medium (20% DMSO in FBS) was added dropwise while continuously gently mixing the content. The cell suspension was immediately dispensed in volumes of 1 ml into pre-chilled labelled 2 ml cryovials (Greiner Bio-one), giving a final concentration of 10×10^6 cells/ml, placed into a 'Mr Frosty' freezing container (Nalgene) and transferred to -80^0C overnight. 'Mr Frostys' contain isopropanol which slows and controls the rate of freezing to -1^0C per minute. The cryovials were then transferred to liquid nitrogen for long term storage (Cryo 200, Forma Scientific).

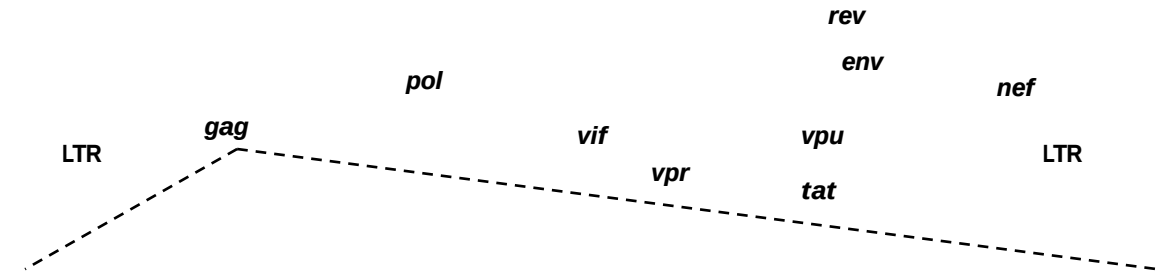
2.8 Thawing of cryo-preserved PBMCs

Thawing PBMCs involves rapid thawing of cells in a 37 ºC water bath until a piece of ice crystal is still visible in the vial. Pre-warmed R1 (1% FBS in RPMI containing 50U penicillin, 50mg/ml streptomycin, 50mg/ml glutamine and 0.8mg/ml Fungin) was added drop

wise while simultaneously mixing the content of the vial. Once the vial was filled with R1, the content was transferred to a 50 ml centrifuge tube which was filled with R1. The cell suspension was carefully mixed by inverting the tube 3 times and was centrifuged at 300 x g for 10 minutes. The supernatant was discarded and cells were washed as before. After washing, the cells were resuspended in the 10ml R20 (20% FBS in RPMI containing 50U penicillin, 50mg/ml streptomycin, 50mg/ml glutamine and 0.8mg/ml Fungin) and rested overnight at 37^{o}C 5% CO_2. Resting PBMCs prior to functional analysis reduces the frequency of non-specific "background" IFN- γ responses measured by ELISPOT probably because cells triggered to undergo apoptosis following thawing would have died before the functional assay is performed (Letsch *et al.*, 2003).

2.9 Preparation of HIV-1 Subtype C Gag and Nef peptide pools

HIV-1 subtype C Du 422 Gag and consensus Nef peptides were kindly provided by the NIH AIDS Reagent Program and Dr Clive Gray (National Institute of Communicable Diseases, Johannesburg, South Africa), respectively. These HIV subtype C peptide sets are based on or are closely related to the consensus sequence of HIV-1 Gag and Nef subtype C proteins circulating in South Africa. A total of 121 15-mer Gag peptides and 50 15-mer Nef peptides overlapping by 11 amino acids (Figures 2.2 and 2.3), which spanned the entire Gag and Nef proteins were used in this study. These peptides were obtained in a lyophilised form and were reconstituted in 50µl DMSO (Sigma) to provide a final peptide concentration of 20mg/ml which were stored in 10µl aliquots at -80°C. From the Gag and Nef stocks, individual peptides were combined to form 5 pools and 33 matrices of Gag (Table 2.1) and 5 pools and 10 matrices of Nef (Table 2.2). These combined peptide pools and matrices were diluted with RPMI to a working concentration of 40µg/ml and arranged into pool/matrix sets stored at -80^{o}C. This design allowed for each peptide to be represented twice within an ELISPOT assay, once in the pool as well as once in the matrix. This design also allowed each peptide to be tested without the need for 121 individual Gag and 50 individual Nef stimulations. Pool 1 spanned HIV-1 Gag p17 (matrix protein); pool 2, 3 and 4 spanned the highly conserved HIV-1 Gag p24 protein (capsid protein) and pool 5 spanned HIV-1 Gag p15 protein (nucleocapsid) as shown in Figure 2.2. The Nef pool distribution is shown in Figure 2.3.



p17 - MATRIX	p24 - CAPSID			p15 - NUCLEOCAPSID
Pool 1	Pool 2	Pool 3	Pool 4	Pool 5
MAARASILRGEKLDK	ADGKVSQNYPIVQNL	MLKDTINEEAAEWDR	VSILDIRQGPKEPFR	PGHKARVLAEAMSQT
ASILRGEKLDKWEKI	VSQNYPIVQNLQGQM	TINEEAAEWDRLHPV	DIRQGPKEPFRDYVD	ARVLAEAMSQTNSGN
RGEKLDKWEKIRLRP	YPIVQNLQGQMVHQA	EAAEWDRLHPVHAGP	GPKEPFRDYVDRFFK	AEAMSQTNSGNIMMQ
LDKWEKIRLRPGGKK	QNLQGQMVHQAISPR	WDRLHPVHAGPIAPG	PFRDYVDRFFKTLRA	SQTNSGNIMMQRSNF
EKIRLRPGGKKHYML	GQMVHQAISPRTLNA	HPVHAGPIAPGQMRE	YVDRFFKTLRAEQAT	SGNIMMQRSNFKGPR
LRPGGKKHYMLKHIV	HQAISPRTLNAWVKV	AGPIAPGQMREPRGS	FFKTLRAEQATQEVK	MMQRSNFKGPRIVK
GKKHYMLKHIVWASR	SPRTLNAWVKVIEEK	APGQMREPRGSDIAG	LRAEQATQEVKNWMT	SNFKGPRRIVKCFNC
YMLKHIVWASRELER	LNAWVKVIEEKAFSP	MREPRGSDIAGTTST	QATQEVKNWMTDTLL	GPRRIVKCFNCGKEG
HIVWASRELERFALN	VKVIEEKAFSPEVIP	RGSDIAGTTSTLQEQ	EVKNWMTDTLLVQNA	IVKCFNCGKEGHIAR
ASRELERFALNPGLL	EEKAFSPEVIPMFTA	IAGTTSTLQEQIAWM	WMTDTLLVQNANPDC	FNCGKEGHIARNCRA
LERFALNPGLLETSE	FSPEVIPMFTALSEG	TSTLQEQIAWMTSNP	TLLVQNANPDCKTIL	KEGHIARNCRAPRKK
ALNPGLLETSEGCKQ	VIPMFTALSEGATPQ	QEQIAWMTSNPPIPV	QNANPDCKTILRALG	IARNCRAPRKKGCWK
GLLETSEGCKQIMKQ	FTALSEGATPQDLNT	AWMTSNPPIPVGDIY	PDCKTILRALGPGAT	CRAPRKKGCWKCGKE
TSEGCKQIMKQLQPA	SEGATPQDLNTMLNT	SNPPIPVGDIYKRWI	TILRALGPGATLEEM	RKKGCWKCGKEGHQM
CKQIMKQLQPALQTG	TPQDLNTMLNTVGGH	IPVGDIYKRWIILGL	ALGPGATLEEMMTAC	CWKCGKEGHQMKDCT
MKQLQPALQTGTEEL	LNTMLNTVGGHQAAM	DIYKRWIILGLNKIV	GATLEEMMTACQGVG	GKEGHQMKDCTERQA
QPALQTGTEELKSLY	LNTVGGHQAAMQMLK	RWIILGLNKIVRMYS	EEMMTACQGVGGPGH	HQMKDCTERQANFLG
QTGTEELKSLYNTVA	GGHQAAMQMLKDTIN	LGLNKIVRMYSPVSI	TACQGVGGPGHKARV	DCTERQANFLGKIWP
EELKSLYNTVATLYC	AAMQMLKDTINEEAA	KIVRMYSPVSILDIR	GVGGPGHKARVLAEA	RQANFLGKIWPSHKG
SLYNTVATLYCVHEK		MYSPVSILDIRQGPK		FLGKIWPSHKGRPGN
TVATLYCVHEKIEVR				IWPSHKGRPGNFLQN
LYCVHEKIEVRDTKE				HKGRPGNFLQNRPEP
HEKIEVRDTKEALDK				PGNFLQNRPEPTAPP
EVRDTKEALDKIEEE				LQNRPEPTAPPAESF
TKEALDKIEEEQNKC				PEPTAPPAESFRFEE
LDKIEEEQNKCQQKT				APPAESFRFEETTPA
EEEQNKCQQKTQQAK				ESFRFEETTPAPKQE
NKCQQKTQQAKAADG				FEETTPAPKQEPIER
QKTQQAKAADGKVSQ				TPAPKQEPIEREPLT
QAKAADGKVSQNYPI				KQEPIEREPLTSLKS
				IEREPLTSLKSLFGS
				PLTSLKSLFGSDPLS
				LKSLFGSDPLSQ

Figure 2.2 Alignment of Gag subtype C Du422 peptide pools across the Gag protein within the HIV genome. Gag regions represented are Gag p17 (matrix), Gag p24 (capsid) and Gag p15 (nucleocapsid) split into a total of 5 pools. The 121 peptides which span HIV Gag are 12-15 amino acids (aa) in length and each peptides overlap by 11 aa. The peptide portions shown in black letters represent the overlapping amino acids and the non-overlapping amino acids are shown in coloured letters.

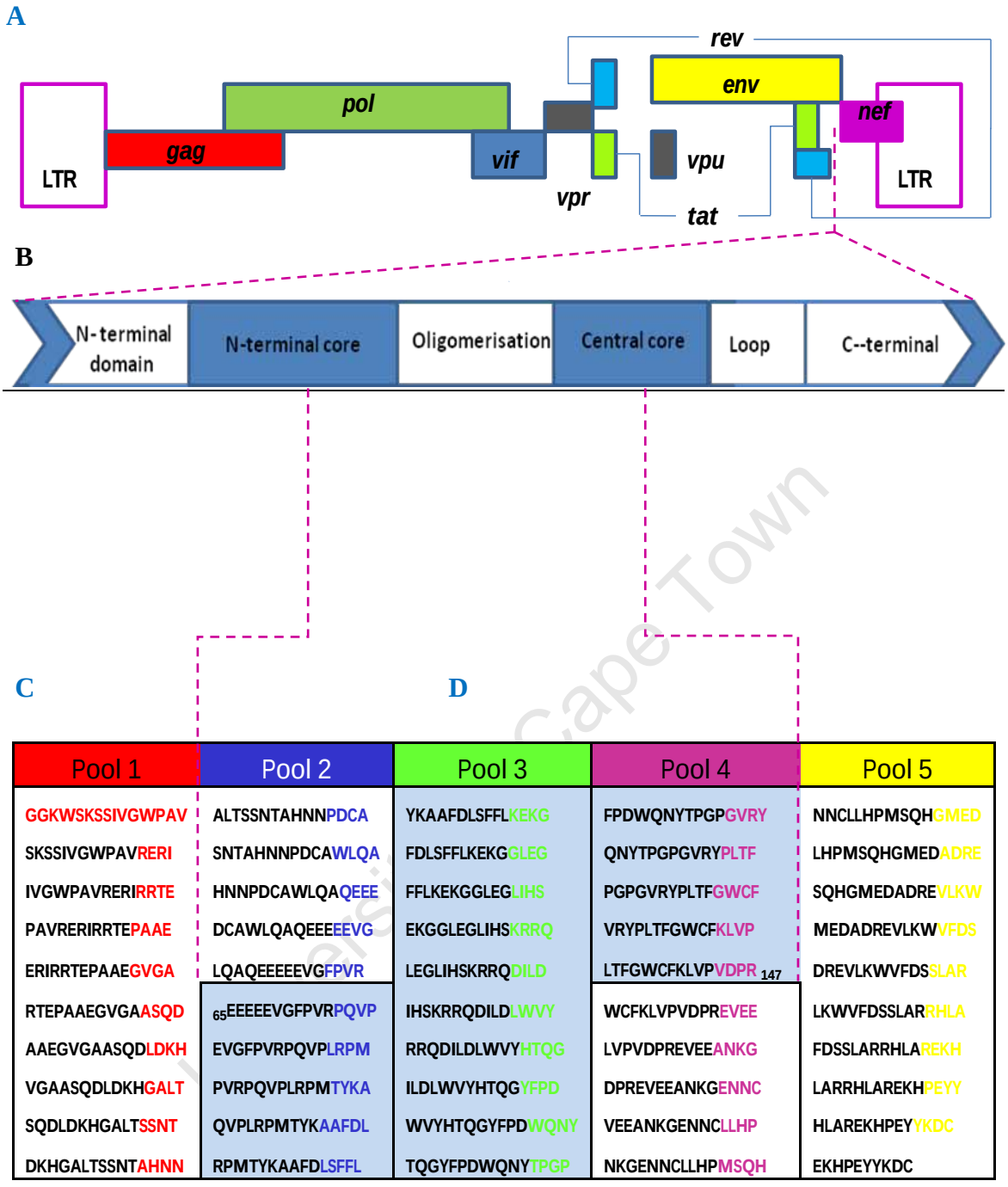


Figure 2.3 Alignment of Nef subtype C peptide pools across the Nef protein within the HIV genome. [A] The proteins of the HIV genome. [B] The domains of the HIV Nef protein. [C] A total 50 amino acid sequences which constitute the 5 peptide pools that span the Nef protein. The peptide length was 15 ± 1 aa overlapping by 11aa indicated by the amino acids in black. [D] The peptide sequences that span the central conserved region of $Nef_{aa65-147}$ as represented by the blue highlighted region.

Table 2.1 HIV-1 Gag peptide pool and matrix design

	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11
P1	1	2	3	4	5	6	7	8	9	10	11
P2	31	32	33	34	35	36	37	38	39	40	41
P3	50	51	52	53	54	55	56	57	58	59	60
P4	70	71	72	73	74	75	76	77	78	79	80
P5	89	90	91	92	93	94	95	96	97	98	99

	M12	M13	M14	M15	M16	M17	M18	M19	M20	M21	M22
P1	12	13	14	15	16	17	18	19	20	21	22
P2	42	43	44	45	46	47	48	49			
P3	61	62	63	63	65	66	67	68	69		
P4	81	82	83	84	85	86	87	88			
P5	100	101	102	103	104	105	106	107	108	109	110

	M23	M24	M25	M26	M27	M28	M29	M30	M31	M32	M33
P1	23	24	25	26	27	28	29	30			
P2											
P3											
P4											
P5	111	112	113	114	115	116	117	118	119	120	121

The highlighted [ ] peptides = pool 1 spans HIV-1 Gag p17; [ ] peptides = pool 2, [ ] peptides = pool 3 and [ ] peptides = pool 4. Pools 2, 3 and 4 span the HIV-1 Gag p24.The [ ] peptides = pool 5 which span HIV-1 p15. An example of composition of a pool matrix; Pool 1 (P1) = 30 peptides, 1-30 (Squares going across); matrix 1 (M1) = 5 peptides, peptides 1, 31, 50, 70 and 89 (squares going down).

Table 2.2 HIV-1 Nef peptide pool and matrix design

	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10
P1	1	2	3	4	5	6	7	8	9	10
P2	11	12	13	14	15	16	17	18	19	20
P3	21	22	23	24	25	26	27	28	29	30
P4	31	32	33	34	35	36	37	38	39	40
P5	41	42	43	44	45	46	47	48	49	50

The highlighted [ ] peptides = pool 1; [ ] peptides = pool 2, [ ] peptides = pool 3; - [ ] peptides = pool 4 and [ ] peptides = pool 5. An example of composition of a pool matrix; - Pool 1 (P1) comprised peptides 1-10 (Squares going across) and matrix 1(M1) comprised of peptides 1,11,21,31 and 41 (Squares going down).

2.10 Preparation of Cytomegalovirus-Epstein Barr virus-Influenza virus (CEF) peptide pools

Cytomegalovirus (CMV), **E**pstein Barr virus (EBV) and In**f**luenza virus (Flu) [CEF] peptides comprise a pool of immunodominant peptides containing common HLA (human leukocyte antigen)-restricted T cell epitopes from each of these viruses (Currier *et al*., 2002). CEF peptides are useful positive controls for inclusion in ELISPOT assays because they have been shown to stimulate the production of IFN-γ from CD8+ T cells in individuals with HLA types common in most populations and most individuals are positive for at least one of these common viral infections (Currier *et al*., 2002). These lyophilised peptides were kindly provided by Dr Clive Gray (NICD) and the NIH AIDS Reagent Program. CEF peptides were initially reconstituted with 50 µl DMSO to form stock solution at a concentration of 20mg/ml which was stored at -80^{0}C. From these stocks, RPMI was added to form working stock concentrations of 40ug/ml and were kept at -80^{0}C. CEF was used at a final concentration of 1µg/ well.

2.11 Preparation of Phytohaemagglutinin (PHA)

Phytohaemagglutinin (PHA; Sigma) is a mitogen which causes most cells to produce IFN-γ or proliferate and is therefore useful as a positive control. PHA stocks (1mg/ml) were stored in 60µl aliquots at -20^{0}C. PHA was used at a final concentration of 16 µg/ ml in the ELISPOT assay.

2.12 IFN-γ ELISPOT assay

The IFN-γ ELISPOT assay quantifies the number of T cells that produce IFN-γ in response to stimulation with recall antigen or mitogens (Streeck *et al*., 2009). In this study, ELISPOT was performed as previously described (Bere *et al.*, 2010) using fresh PBMCs from the acute HIV infection cohort and frozen PBMCs from the long-term non-progressor cohort (described in sections 2.5 and 2.7). Initially, 96-well polyvinylidene diflouride (PVDF) plates (Multiscreen IP, Millipore) were coated with 50µL anti-IFN-γ monoclonal antibody clone 1-D1k (Mabtech) at a final concentration of 2µg/ml in PBS. Plates were sealed with a plastic cover to prevent evaporation of the antibody and stored overnight at 4ºC. Unbound antibodies were removed by washing each well three times with 200µl PBS. In order to prevent non-specific binding of the secondary antibodies to the membrane, unbound sites

were blocked by incubating the plate with 200µl RPMI containing 10% FCS as a source of blocking protein for 2 hours at room temperature. To each well of the coated plate, 50 µl of Gag, Nef or CEF peptides diluted in R10 at a final concentration of 1µg per ml were added, followed by 50 µl of PBMCs containing 10^5 cells ($2x10^6$/ml of R10). The plating procedure of cells and peptides was done according to the representative plate layout shown in Table 2.3. Positive controls [including CEF peptides (final concentration of 1µg/well) and PHA (final concentration of 16 µg/ml) and negative controls [including medium alone and unstimulated PBMCs (background)] were included on each plate. In addition, PBMCs from a donor with well-characterized CEF responses were included on each ELISPOT plate as a quality control across different plates and different assays (described in detail in Appendix 2).

Plates containing PBMCs and peptides were incubated at 37ºC 5% CO_2 for 18- 24 hours. At the end of incubation, plates were washed six times with 0.01M PBS 0.05% Tween (Sigma) with an automated plate washer (ELX50, Bio-tek Instruments). After washing, 50 µl biotinylated anti–IFN-γ monoclonal antibody (2µg/ml in 10% FBS/PBS; Mabtech) was added to each well and incubated for 2 hours at room temperature in the dark. The plates were washed six washes with PBS Tween as before. Subsequently 100 µl of Streptavidin horseradish peroxidase (Pharmigen) at a concentration of 2µg/ml in 10% FBS/PBS were added to each well and the plates were incubated for another hour at room temperature. The plates were again washed six times with 0.01M PBS 0.05% Tween. Finally, the IFN-γ spots were developed by the addition of 100 µL NovaRED substrate (Vector Laboratory) for 6 minutes in the dark.

The reaction was stopped by rinsing the plates with running tap water and plates were left to dry overnight in the dark. Developed spots were quantified using the automated ImmunoSpot reader (Cellular Technology Ltd.) using ImmunoSpot Version 3 software. After the spots were automatically quantified, a printout was generated which reflected a picture of the ELISPOT plate and the number of spots were automatically recorded next to each well (Figure 2.4).

Table 2.3 Representative figure of ELISPOT pipetting plan showing arrangement of Gag and Nef pools and matrices.

	1	2	3	4	5	6	7	8	9	10	11	12
A	MED	GP1	GP2	GP3	GP4	GP5	GM1	GM2	GM3	GM4	GM5	GM6
B	GM7	GM8	GM9	GM10	GM11	GM12	GM13	GM14	GM15	GM16	GM17	GM18
C	GM19	GM20	GM21	GM22	GM23	GM24	GM25	GM26	GM27	GM28	GM29	GM30
D	GM31	GM32	GM33	NP1	NP2	NP3	NP4	NP5	NM1	NM2	NM3	NM4
E	NM5	NM6	NM7	NM8	NM9	NM10						
F	BG	BG	BG			PHA	PHA	PHA		CEF	CEF	CEF

Each square represents a well of a 96 well plate. HIV Gag (blue blocks) and Nef (green blocks) peptides were used in the acute HIV infection study while only Gag peptides were used in LTNP HIV infection study. All peptides were represented twice, once in the pools and once in the matrix. MED = Medium; GP= Gag Pool; NP = Nef pool; BG = Background; PHA = Phytohaemagglutinin and CEF = Cytomegalovirus, Epstein-Barr virus, influenza virus (yellow blocks).

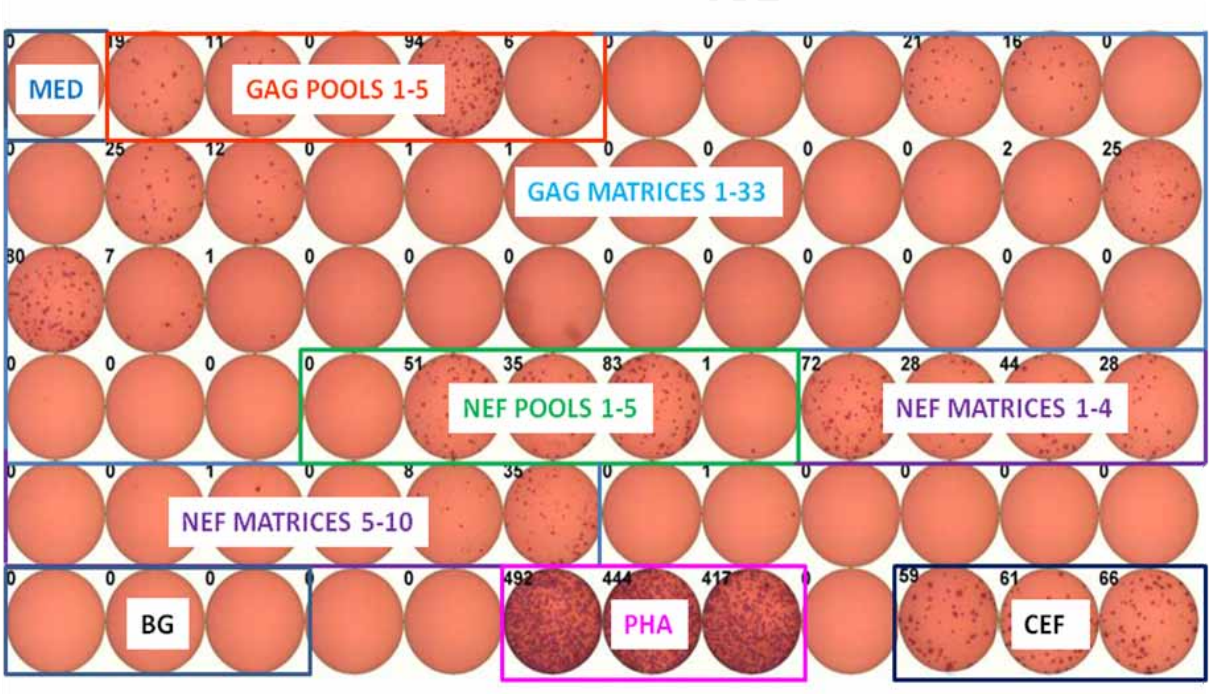


Figure 2.4 A representative IFN-γ ELISPOT plate showing the number of spots counted next to each well generated by the automated Immunospot reader. Each well corresponds to the square of the layout presented in Table 2.3. From this result, spot forming units (SFUs) per well can be calculated by subtracting the background spots from the spots in the test wells (Table 2.4). In this test sample there was no background spots.

2.13 Calculation of IFN-γ spot forming units (SFUs) and cut-offs

To quantify IFN-γ T cell responses, the spots were expressed in a unit that enables the comparison of the magnitude and breadth of IFN-γ responses to HIV antigens Gag and Nef. ELISPOT spot forming unit (SFUs) per 10^6 PBMCs were calculated by subtracting the mean background number of spots obtained in negative control wells from the number of spots obtained in the test wells. After background correction, SFUs were multiplied by a factor of 10 since 10^5 cells were plated per well and the final results were expressed in SFU/10^6 PBMC.

For the acute cohort, in which PBMCs were assayed fresh, a net IFN-γ response of≥50 SFU/10^6 PBMCs was used as the cut-off for considering a response positive. In support of this, Addo *et al*. (2002), Kaufmann *et al*. (2004) and Turk *et al*. (2008) have previously defined a positive response by ELISPOT as being >50 SFU/10^6 cells. For the LTNP cohort, in which PBMCs were frozen before assessment, a response of≥100 SFU/10^6 PBMC above background was regarded as positive as previously determined by Liebenberg (2007). Using the ELISPOT results from a chronically HIV-infected woman shown in Figure 2.4, Table 2.4 demonstrates how SFUs/10^6 cells were calculated from spots generated by the automatic scanner.

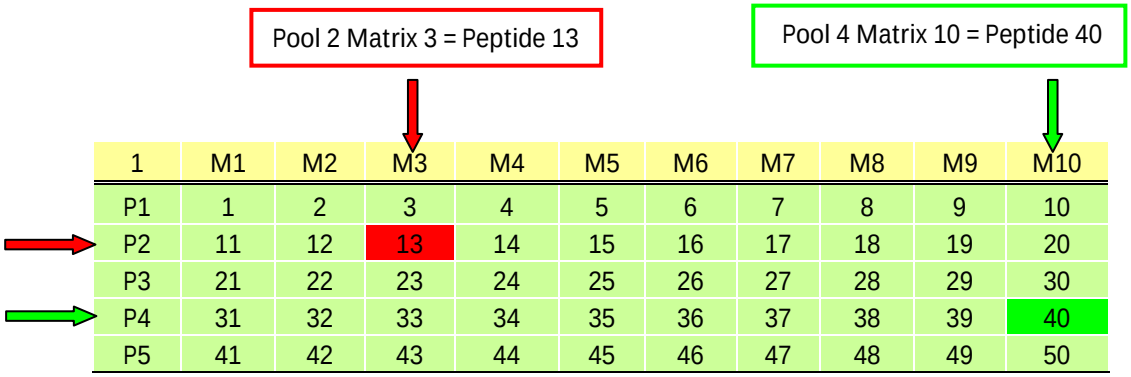
2.14 Confirmatory ELISPOT of individual Gag and Nef peptide specificities

Although the Gag and Nef peptide pool–matrix approach (Figure 2.4) to mapping individual HIV specificities identifies putative individual peptide specificities, it was necessary to confirm these specificities using a subsequent ELISPOT in which the individual peptides identified in the screen were included. Each peptide pool with a positive response (defined in section 2.13) was matched with each matrix with a positive response resulting in the identification of the putative individual peptide/s. This is illustrated in Figure 2.5. Once the potential reactive peptides were identified in this way, individual peptides matching the ones identified were confirmed by a subsequent ELISPOT. For confirmatory ELISPOTS, individual peptides were evaluated in duplicate.

Table 2.4 Method used to calculate SFU/10^6 cells in responses to HIV Gag and Nef pools and matrices from the ELISPOT experiment shown in Figure 2.4.

		Spot no	SFU/10^6 Cells			Spot no	SFU/10^6 Cells
	BG	0					
Gag	POOL 1	19	190	Nef	POOL 1	0	0
	POOL 2	11	110		POOL 2	51	510
	POOL 3	0	0		POOL 3	35	350
	POOL 4	94	940		POOL 4	83	830
	POOL 5	6	60		POOL 5	1	10
Gag	MATRIX 1	0	0	Nef	MATRIX 1	72	720
	MATRIX 2	0	0		MATRIX 2	28	280
	MATRIX 3	0	0		MATRIX 3	44	440
	MATRIX 4	21	210		MATRIX 4	28	280
	MATRIX 5	16	160		MATRIX 5	0	0
	MATRIX 6	0	0		MATRIX 6	0	0
	MATRIX 7	0	0		MATRIX 7	1	10
	MATRIX 8	25	250		MATRIX 8	0	0
	MATRIX 9	12	120		MATRIX 9	8	80
	MATRIX 10	0	0		MATRIX 10	35	350
	MATRIX 11	1	10				
	MATRIX 12	1	10		PHA	492	4920
	MATRIX 13	0	0		PHA	444	4440
	MATRIX 14	0	0		PHA	417	4170
	MATRIX 15	0	0				
	MATRIX 16	0	0		CEF(1425)	59	590
	MATRIX 17	2	20		CEF(1425)	61	610
	MATRIX 18	25	250		CEF(1425)	66	660
	MATRIX 19	80	800				
	MATRIX 20	7	70				
	MATRIX 21	1	10				
	MATRIX 22-33	0	0				

The background response was 0. Background SFU were subtracted from each stimulation condition and then multiplied by 10 (since 10^5 cells were plated/well while SFUs are expressed per 10^6 cells). For this chronically HIV infected women, a positive HIV-specific response was considered to be any response $\geq$ 100 SFU/10^6 and these are highlighted in blue and green. Donor WP 1425 was used as the quality control PBMC sample included on every ELISPOT experiment performed. CEF responses (yellow) by donor WP 1425 had to be within the range of 337-776 SFU/10^6 cells (detailed in appendix 1) and PHA(yellow) responses > 500 SFU/10^6 (Streeck *et al.*, 2009) cells for an experiment to be considered valid.



1	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10
P1	1	2	3	4	5	6	7	8	9	10
P2	11	12	13	14	15	16	17	18	19	20
P3	21	22	23	24	25	26	27	28	29	30
P4	31	32	33	34	35	36	37	38	39	40
P5	41	42	43	44	45	46	47	48	49	50

Figure 2.5 Method used to identify two putative individual peptides detected in the chronically HIV-1 infected sample from positive responses in distinct pools and matrices. The combination of a positive response to NEF pool 2 and matrix 3 would predict that the individual Nef peptide 13 was being recognized. The combination of Nef pool 4 and matrix 10 would predict that the individual Nef peptide 40 was being recognized.

Positive responses may be detected in adjacent overlapping peptides therefore, when consecutive peptides were positive on the pool/matrix screening ELISPOT, only the higher of the two responses was considered positive. In the case of three consecutive peptides being positive, the highest two responses were considered positive.

2.15 Quality controls included on each ELISPOT plate

The ELISPOT assay is a robust, reliable and reproducible way to detect IFN-γ T cell responses (Gray *et al.*, 2009). It is, however, important to include quality control measures to ensure that consistency is maintained throughout the study and across plates. In this study, CEF peptides and PHA were used as positive controls; while medium alone and medium with cells were used as negative controls (as represented in Figure 2.4). IFN-γ responses generated from negative control wells were never >30 SFU/10^6 PBMC. While PHA was used to test the maximum ability of PBMCs to produce IFN-γ, CEF peptides served as a positive viral antigen control. In addition, on each plate, PBMCs derived from a donor with well characterized responses to CEF peptides were included as a quality control for interplate and inter assay variability. CEF quality control PBMCs were used on each ELISPOT plate included in this study and the details of this approach are detailed in Appendix 1.

2.16 Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 (San Diego California USA). Mann-Whitney U test and the Wilcoxon Ranks Test were applied for matched and non-matched non-parametric comparisons, respectively. The Spearman Ranks and Pearson correlation were applied for testing correlations between non-parametric and parametric data sets, respectively. In all experiments, a two-tailed p-value of ≤ 0.05 was considered significant.

CHAPTER 3

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3.1 Clinical characteristics of acutely HIV-infected individuals

Of the 500 HIV negative individuals recruited into this prospective observational sero-incidence study, 7 became infected with HIV during the 12 months of follow-up. The schedule of evaluation for each of the seven individuals who became infected with HIV is shown in Table 3.1. At enrollment (Table 3.2), the mean age of acutely infected individuals was 26 years (± 9 years; SD), they had a mean HIV viral load of 43004 RNA copies/ml (± 82174; SD), and a mean CD4 count of 504 cells/µl (± 259; SD). All 7 individuals who became infected with HIV during follow-up were female. Acute HIV infection was considered to be the incident visit (mean of 21 days post infection; ± 13 days) while 1-3 months post-infection was considered early infection and >6 months was considered to be chronic infection. All the participants were confirmed by sequencing Gag to be infected with HIV-1 subtype C by Michelle Skelton in Prof Carolyn Williamson's laboratory at the University of Cape Town (data not shown).

Table 3.1 Monthly clinical visits of acutely (HIV-1 infected women

Gender	PID	Incident	M1	M2	M3	M6	M12	M18	M24	M30
F	C001									
F	C002									
F	B349									
F	B420									
F	C004									
F	C007									
F	C008									

Green bars indicate the gender; yellow bars indicate their study number and blue bars, the clinical visits in months.

Table 3.2 Clinical characteristics of women acutely infected with HIV

PID[a]	Age	HIV-1 infection at enrollment	HIV-1 load	CD4 count	Duration of follow up
	(Years)	(Days)	(RNA copies/ml)	(cells/µl)	(Months)
C001	16	14	NA[b]	548	30
C002	25	45	7480	548	4
C004	37	36	11000	867	24
C007	17	14	228000	177	3
C008	21	15	16900	787	18
B349	34	15	6400	305	0
B420	34	11	31200	298	0
Mean(SD)	26 ± 9	21 ± 13	43004 ± 82174	504 ± 259	11 ± 12

[a] PID (participant identification); [b] not available

CD4 counts during acute HIV infection were lower than those measured during early or chronic infection time points in women with longitudinal samples available although this was not significant (Figure 3.1A). No significant difference was observed between plasma viral loads measured during acute, early and chronic phases of infection (Figure 3.1B). Despite being recruited at 21 days post HIV-infection, this finding suggests that their acute phase peak viral load was already declining at the earliest time point (incident visit) at which their viral loads were measured. There was, however, no association between time post-HIV infection (estimated time from infection to enrollment in days) and plasma viral load (data not shown).

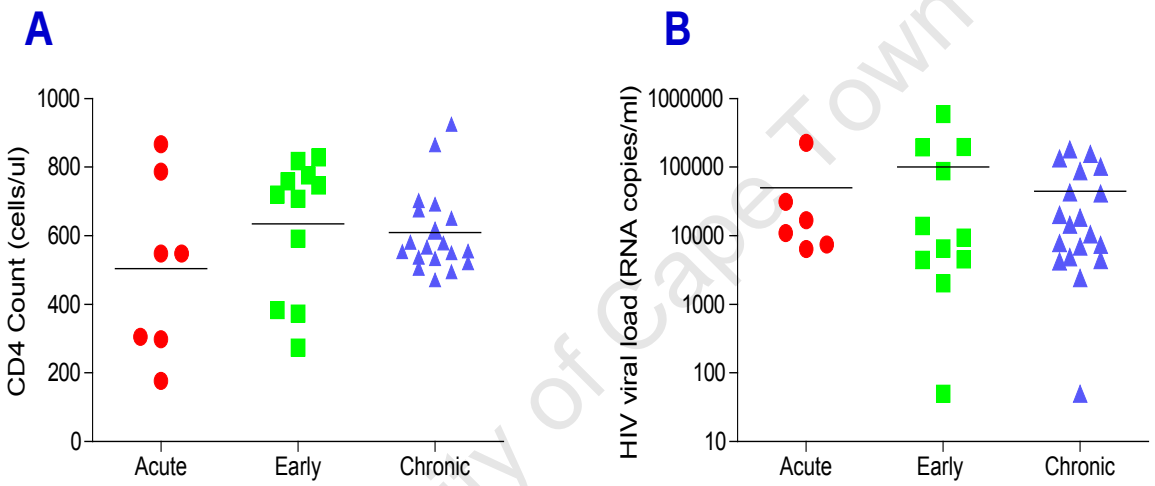


Figure 3.1 Blood CD4 counts (A) and HIV RNA loads in plasma (B) in acutely HIV-infected women. Red spots represent acute (incident); green squares represent early infection (1-3 months); and blue triangles represent chronic infection (>6 months). Mann-Whitney U tests were applied to compare these non-parametric variables. All data points reflect the CD4 counts and HIV RNA loads represented in tables A1 and A2 in the appendices section.

3.2 Relationship between CD4 T cell counts in blood and plasma viral load during acute HIV infection

In acutely HIV-infected women over time, CD4 T cell counts were inversely associated with plasma viral load (Figure 3.2; Rho = -0.38; p=0.02). Figure 3.3 shows the longitudinal relationship between plasma viral load and CD4 T cell counts in 5 of the 7 acutely HIV-infected women who were studied longitudinally. Of the 5 women studied longitudinally, the clinical progression for 4 women (C001, C004, C002 and C007) showed CD4 T cell counts in blood declined during the course of infection while plasma viral loads generally increased over time (Figure 3.3). In contrast, C008 showed no relationship between plasma viral load and CD4 T cell counts in blood.

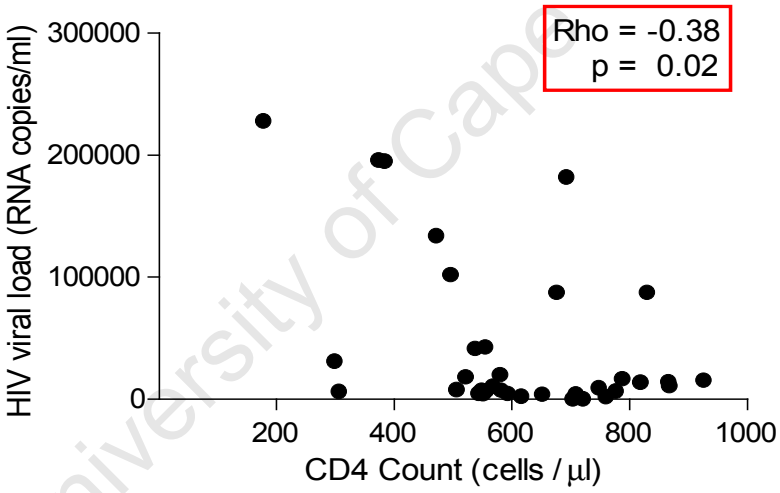


Figure 3.2 Correlation between plasma HIV viral load (RNA copies/ml) and CD4 counts (cells/µl) during acute HIV-infection and over time. Each dot represents the matched measurements of CD4 counts and viral load of participants longitudinally. Spearman correlations were used and $p < 0.05$ were considered as significant.

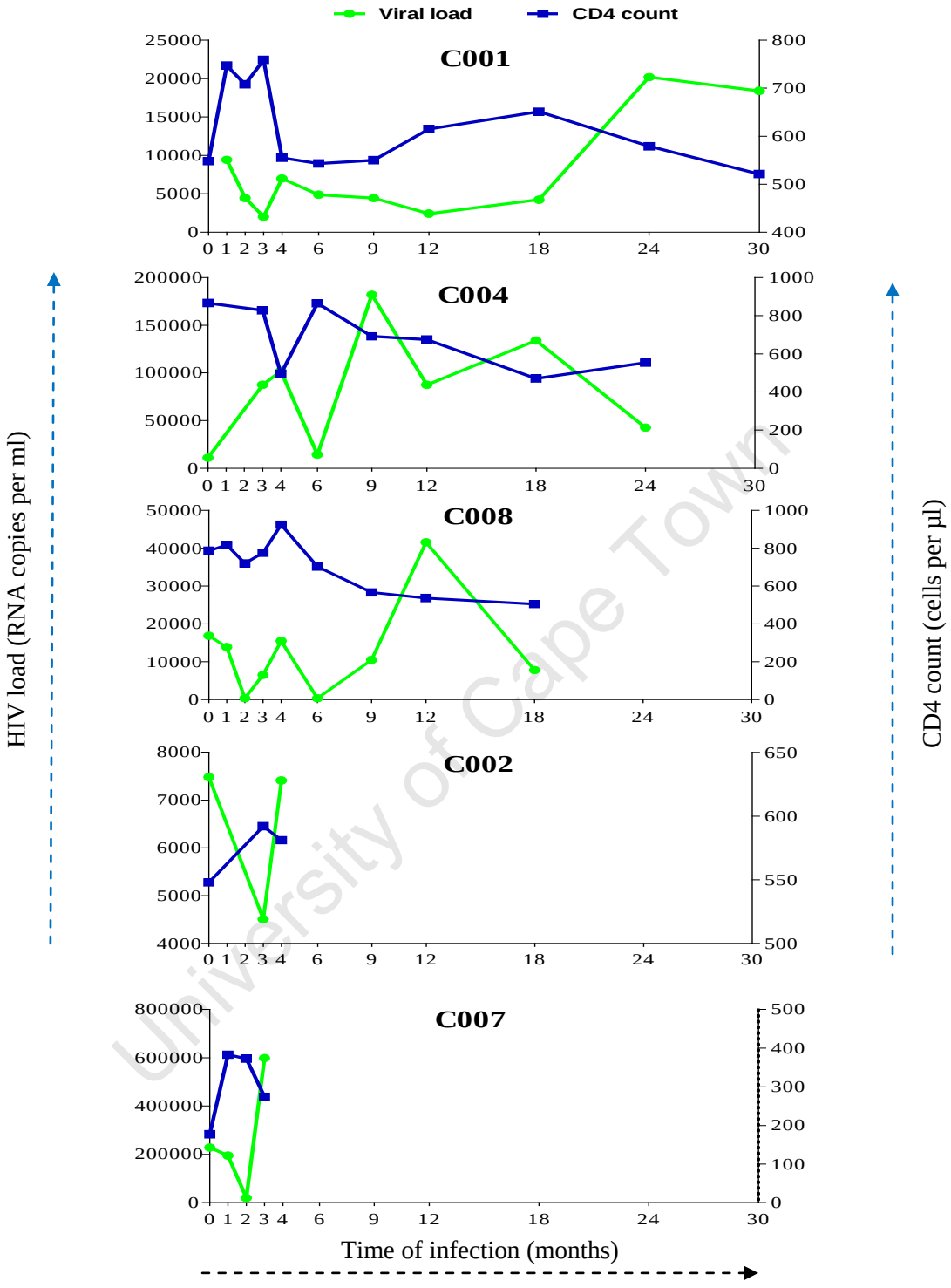


Figure 3.3 Kinetics of CD4 T cell counts in blood (cells/µl) and HIV-1 viral load (RNA copies/ml) in women who became acutely infected with HIV. HIV viral load is indicated by the green dots and CD4 counts are indicated by blue squares. Participant C001 was followed for 30 months, C002 for 4 months, C004 for 24 months, C007 for 3 months and C008 for 18 months. CD4 count and viral load measurements were taken monthly for the first 4 months, 3 monthly from 6 to 12 months and then 6 monthly thereafter.

3.3 Characterization of HIV-1 specific T cell responses during the acute phase of HIV infection

The magnitude and breadth of HIV-1 Gag and Nef-specific T cell responses in acutely HIV-infected women were characterized by IFN-γ ELISPOT. Five out of the seven (71%) acutely HIV-infected women had detectable Gag and/or Nef-specific T cell responses at enrollment (mean 21 days post infection) (Figure 3.4). The magnitude of T cell responses directed against HIV subtype C Gag peptides ranged from 60 – 110 SFU/10^6 PBMC (Figure 3.4A) while responses directed against Nef peptides ranged from 170 – 1110 SFU/10^6 cells (Figure 3.4B). Participant C001 was found to have the highest magnitude response to Gag (200 SFU/10^6 cells) that was directed against Gag pools 1 and 5. Participant B420 had the highest magnitude response to Nef (1110 SFU/10^6 cells) and was the only individual who responded to both Nef and Gag. More acutely infected women targeted Gag but higher magnitude responses were against Nef.

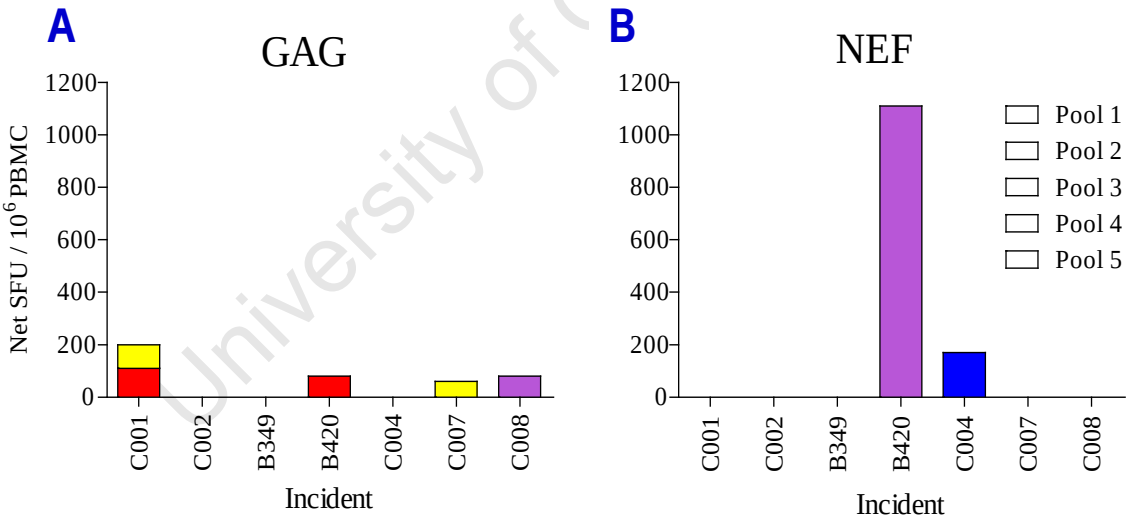


Figure 3.4 Magnitude of HIV-1 specific T cell responses to Gag and Nef peptides during acute infection. Histograms show all positive responses in SFU per million PBMC as determined by the cut off criteria (in method). Graph A represents responses to Gag pools and B responses to Nef pools. Gag pool 1 are peptides representing p17; pool 2, pool 3 and pool 4 the p24 and pool 5 the p15 regions of the HIV-1 Gag protein. Graph B represents the responses to Nef pools. Nef pool 2 and 4 constitute the central region of Nef.

The breadth of HIV-specific T cell responses to Gag and Nef (characterized by the number of Gag or Nef pools targeted) was limited during acute HIV infection with only 2/7 individuals (C001 and B420) targeting more than 1 pool within either Gag or Nef. Participant C001 targeted two Gag pools and B420 targeted 1 Gag and 1 Nef pool. The remaining acutely infected women (C004, C007 and C008) targeted only a single pool within Gag or Nef. There was a statistically significant correlation (Rho = 0.93; p = 0.02) between the number of Gag and/or Nef pools targeted and HIV viral load during the initial stage of disease (results not shown).

The breadth of HIV Gag or Nef responses was further characterized by mapping individual peptide responses in each woman (Table 3.3). Participant C001 targeted peptides Gag11 [LERFALNPGLLETSE] and Gag99 [KEGHIARNCRAPRKK]. Participant B420 targeted Gag7 [GKKHYMLKHIVWASR], Nef33 [PGPGVRYPLTFGWCF] and Nef34 [VRYPLTFGWCFKLVP]. The Nef RW8 epitope [RYPLTFGW], embedded in the Nef 34 peptide [V**RYPLTFGW**CFKLVP] was amongst those epitopes that were confirmed HIV subtype C epitopes from the Los Alamos database (http://www.hiv.lanl.gov/content/hiv-db/ELF/epitope_analyzer.html (accessed 15 December 2010) and matched previously described HIV-1 subtype B epitopes (Masemola *et al*., 2004).

Table 3.3 Individual Gag and Nef peptide mapping during acute HIV infection

PID	Peptide Number	Epitope location	Magnitude (SFU/10^6 cells)	Peptide Sequence	HLA Class I restriction
C001	Gag 11	p17	110*	LERFA**V**NPGLLETSE	B63
	Gag 99	p15	90*	KEGH**L**ARNCRAPRKK	A3
C004	Nef 19	central	410	QVPLRPMTYKAAFDL	B*3501
C007	Gag 99	p15	50	KEGHIA**K**NCRAPRKK	A*03
C008	Gag 74	p24	140	YVDRFFKTLRAEQAT	Cw*0303;B*1503
B420	Gag 7	p17	120	GKKHYMLKHIVWASR	A*2301
	Nef 33	central	360	PGPGVRYPLTFGWCF	B*27
	Nef 34	central	380	VRYPLTFGWCFKLVP	B*27

*Unconfirmed individual peptide specificities. Pool/matrix predicted peptides are included. The underlined portion of the peptide represents the previously defined epitope as described in the Los Alamos database.

The remaining peptides that were targeted by individuals C004, C007 and C008 were Nef19 [QVPLRPMTYKAAFDL], Gag99 [KEGHIARNCRAPRKK], and Gag74 [YDVRFFKTLRAEQAT], respectively. The Nef VY8 epitope [VPLRPMTY], embedded in Nef peptide 19

[Q**VPLRPMTY**KAAFDL], was previously described as an immunodominant epitope in acute infection (Streeck and Nixon, 2010). Although the HLA types of these women were not determined in this study, this Nef VY8 epitope [VPLRPMTY] has previously been reported to be restricted by HLA B*35 and was found to be more likely to be recognized during acute infection than during chronic infection (Streeck and Nixon, 2010). In this study, only 13% of Gag peptides that were targeted during acute HIV infection were located within the most conserved p24 region while the remaining Gag responses were equally located within the p17 and p15 region. All the Nef peptides targeted were situated within the central most conserved region.

3.4 Longitudinal mapping of HIV-specific T cell responses in acutely HIV infected women

Following acute infection, HIV Gag- and Nef-specific T cell responses were characterized over time in 5/7 individuals for whom longitudinal samples were available. Responses were followed until the chronic stages of HIV infection in 3 women (C001, C004 and C008) while responses were followed until early infection in 2 women (C002 and C007). Generally, the total magnitude of T cell responses to Gag and Nef increased over time in all 3 women followed from acute HIV infection to chronic phases of infection (Figure 3.5). T cell responses to Gag dominated the acute phase of infection, whilst responses to Nef were more prominent during the chronic stages of infection. During the first year of infection, the magnitude of Gag in participant C001 fluctuated between 57 and 440 SFU/10^6 cells, reaching a plateau at 800 – 840 SFU/10^6 cells during 18-24 months post infection. In contrast, the Nef response was higher than Gag responses (median 1180 SFU) but fluctuated between 444 and 2400 SFU/10^6 cells. In comparison with C001, the magnitude of Gag responses in participant C004 remained low throughout 24 months of follow-up with the highest response being detected at 12 months (370 SFU/10^6 cells). Nef responses in this woman steadily increased from 170 SFU/10^6 cells during acute HIV infection to 1260 SFU/10^6 cells by 24 months post-infection. In participant C008, T cell responses to both Gag and Nef peaked at 6 months with Gag dominating acute and early infection and Nef dominating chronic infection.

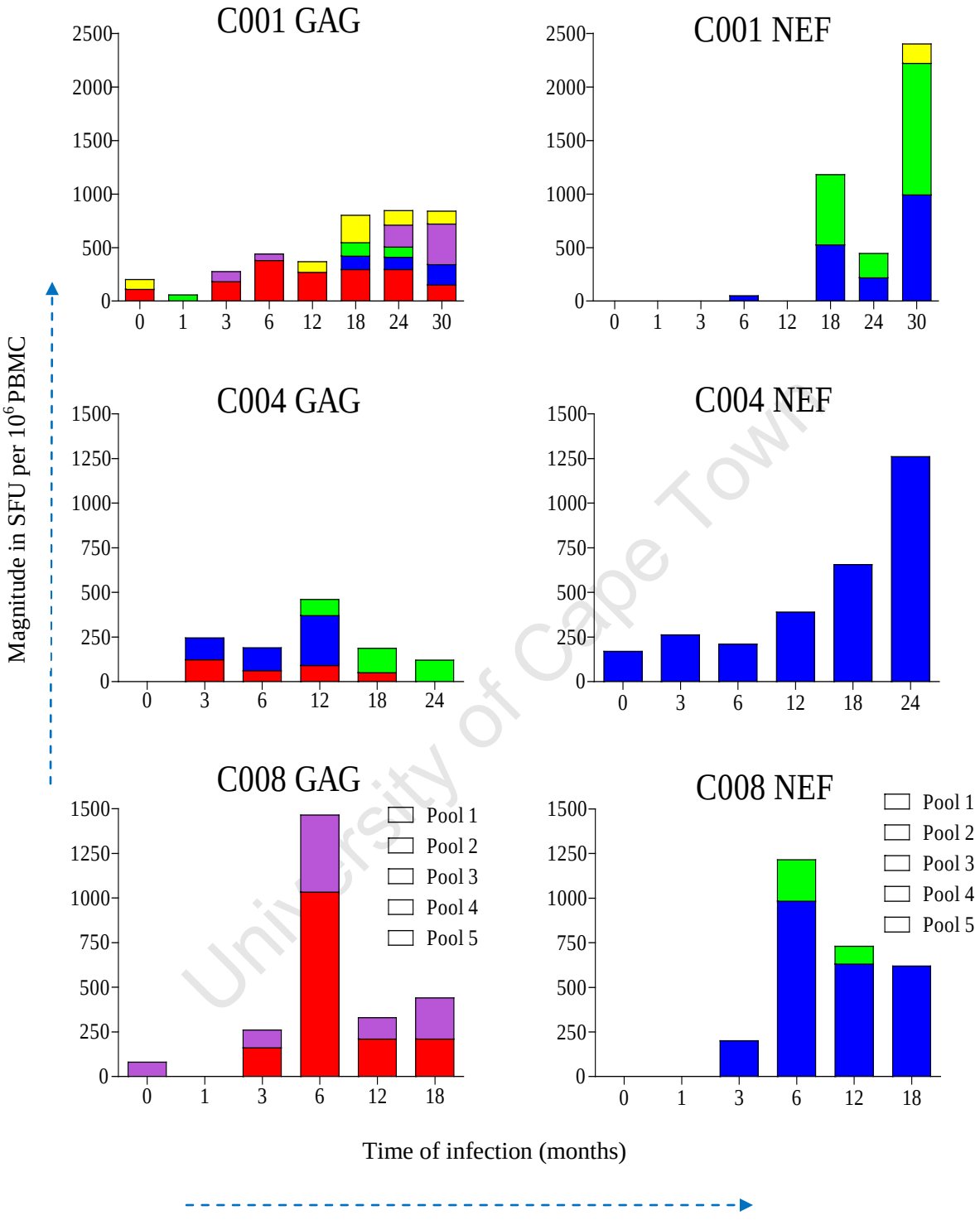


Figure 3.5 Longitudinal HIV-specific T cell responses to Gag and Nef peptides in women who recently became infected with HIV. All responses (SFU/million PBMC) that were determined to be positive were according to the cut off criteria. Gag pool 1 are peptides representing p17 pool 2, pool 3 pool 4 the p24 and pool 5 the p15 regions of the HIV-1 Gag protein. The Nef pools, 2, 3 and 4 represent the central conserved region of Nef.

The extent of expansion and contraction in the total magnitude of Gag and Nef-specific T cell responses were analyzed by assigning a variance score within individual response at each time point. The variance score was calculated by dividing the magnitude of IFN-γ responses (SFU/10^6 cells) measured at a particular time point by the average magnitude at all time points tested. Longitudinal responses with a variance score approaching 1 would be indicative of a stable response over time of infection. The variance score over time for the three (C001, C004 and C008) individuals were unstable during acute infection and appeared more stable as they approached chronic infection (Figure 3.6).

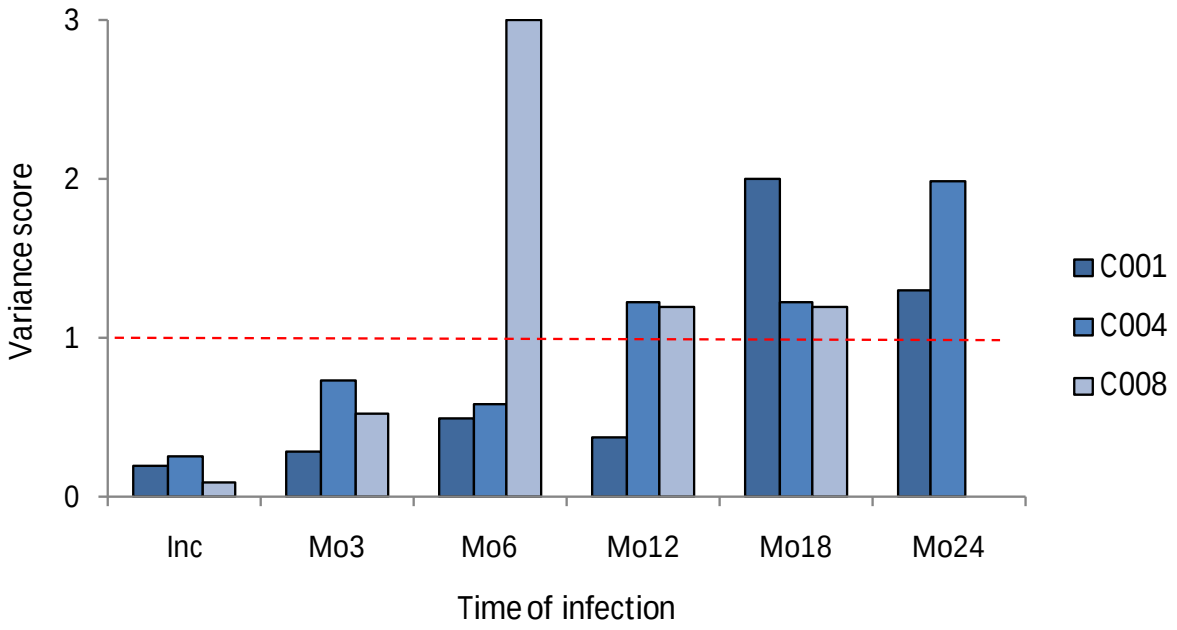


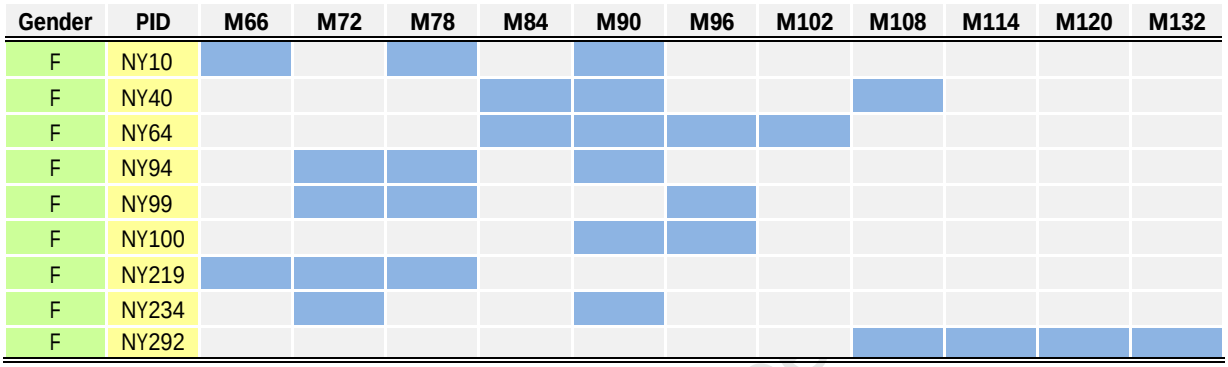
Figure 3.6 Variance scores for Gag and Nef T cell responses measured over 24 months from acute HIV infection in three women with samples available. Participant C001 is shown in dark blue, participant C004 is shown in medium blue, and C008 is shown in light blue. The variance scores were determined by dividing the magnitude to Gag and Nef (SFU/10^6 cells) generated at a particular time point by the average magnitude at all time points tested. A score approaching 1 means low deviation.

3.5 Clinical characteristics of HIV-infected long-term non-progressors

Nine women who had been infected with HIV for >5 years and maintained CD4 counts >300 cell/ul in the absence of HAART were included in this study to compare immune responses measurable in long term non-progressors (LTNPs) with those generated during acute/early HIV infection. These

LTNPs were studied longitudinally at 3-4 consecutive 6 monthly visits and details of the visits included in this study are summarized in Table 3.4. They had been infected for a mean 2352 days or 6.5 years (±412 days; SD) and continued to be naïve to HAART at the time of study (Table 3.5). The mean age of the LTNPs was 35 years (±6 years), and they had a mean HIV viral load of 10170 (±16943; SD) copies per ml.

Table 3.4 Six monthly clinical visits of LTNPs



Green bars indicate gender, yellow bars the study number and blue bars the visits in months

Table 3.5 Clinical characteristics of LTNPs

PID[a]	Age	HIV-1 infection at enrollment	HIV-1 load	CD4 count	Duration of follow up
	(Years)	(Days)	(RNA copies/ml)	(cells/µl)	(Months)
NY010	41	1825	<50	334	24
NY040	41	2373	7200	322	24
NY064	27	2555	930	383	18
NY094	38	2190	1700	465	24
NY099	26	2008	10000	525	36
NY100	33	2373	53000	778	6
NY219	33	2373	16000	551	12
NY234	35	2190	2600	680	24
NY292	42	3285	<50	529	24
Mean(SD)	35 ± 6	2352 ± 412	10170 ± 16943	507 ± 153	21 ± 8

Unlike the women who were acutely infected with HIV, an inverse relationship was not observed between plasma viral load and CD4 counts in the nine LTNPs (Rho= 0.12; p=0.47). Although no relationship was found when all LTNPs were grouped together, four of these nine women (NY040, NY064, NY099 and NY219) showed a non-significant inverse relationship between CD4 counts and

viral load at an individual level while the remaining women (NY010, NY094, NY100, NY234 and NY294) showed no relationship (Figure 3.7 and Figure 3.9). Furthermore, there were no significant differences between longitudinal CD4 counts and HIV-1 plasma viral loads (Figure 3.8A and B).

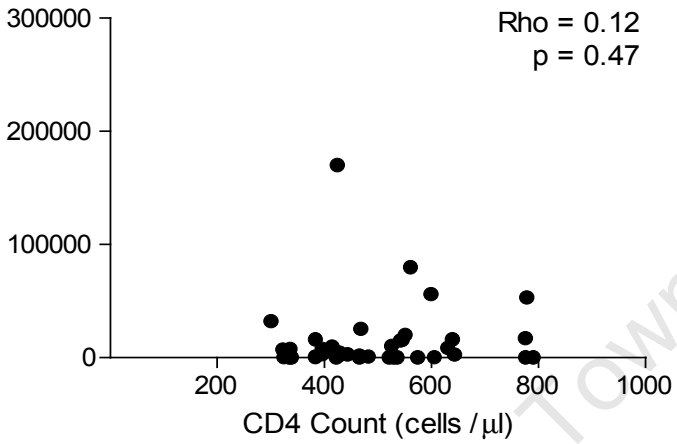


Figure 3.7 Relationship between plasma HIV viral load (RNA copies/ml) and CD4 counts (cells/µl) in HIV-1 infected LTNPs. Each dot represents the matched measurements of CD4 counts and viral load of nine participants longitudinally. Spearman Ranks test for correlation was used to calculate the Rho and p values and a p value of <0.05 was considered to be significant.

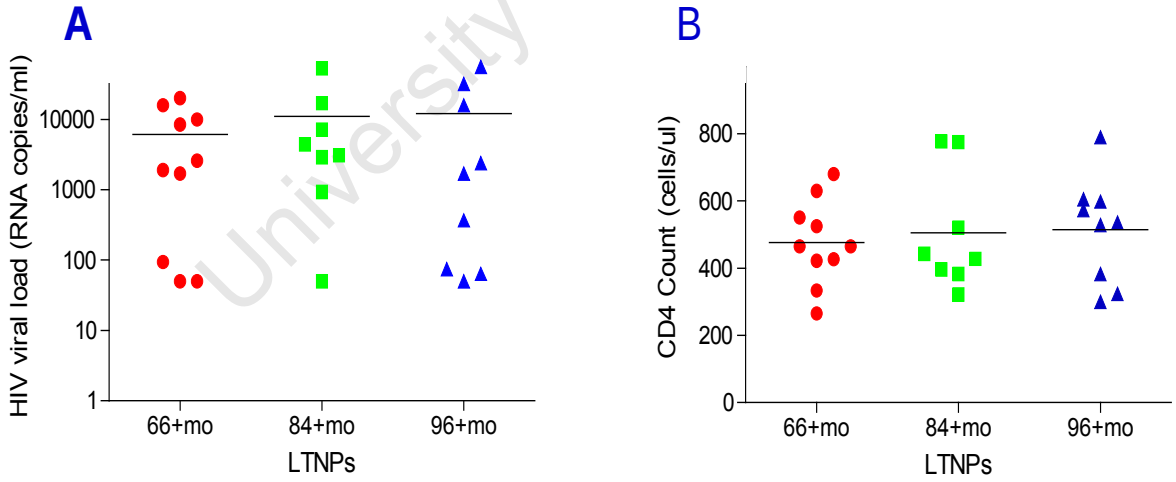
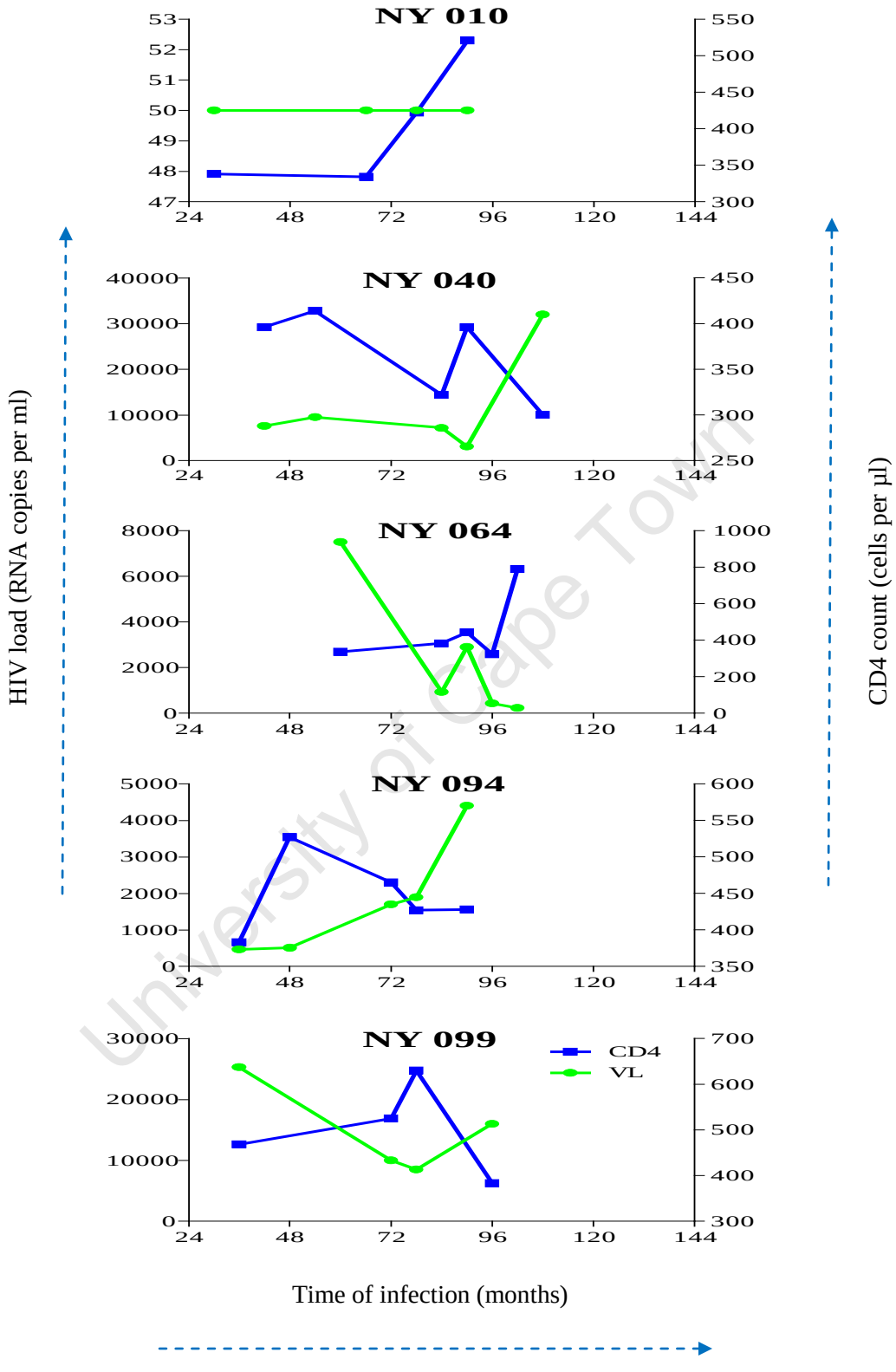


Figure 3.8 Blood HIV-I RNA load (A) and CD4 T cell counts (B) in HIV-infected LTNPs. Red dots represent 66+ months post infection; green squares represent 84+ months post infection; and blue triangles represent 96+ months post infection. Mann-Whitney U tests were applied to compare these non-parametric variables.



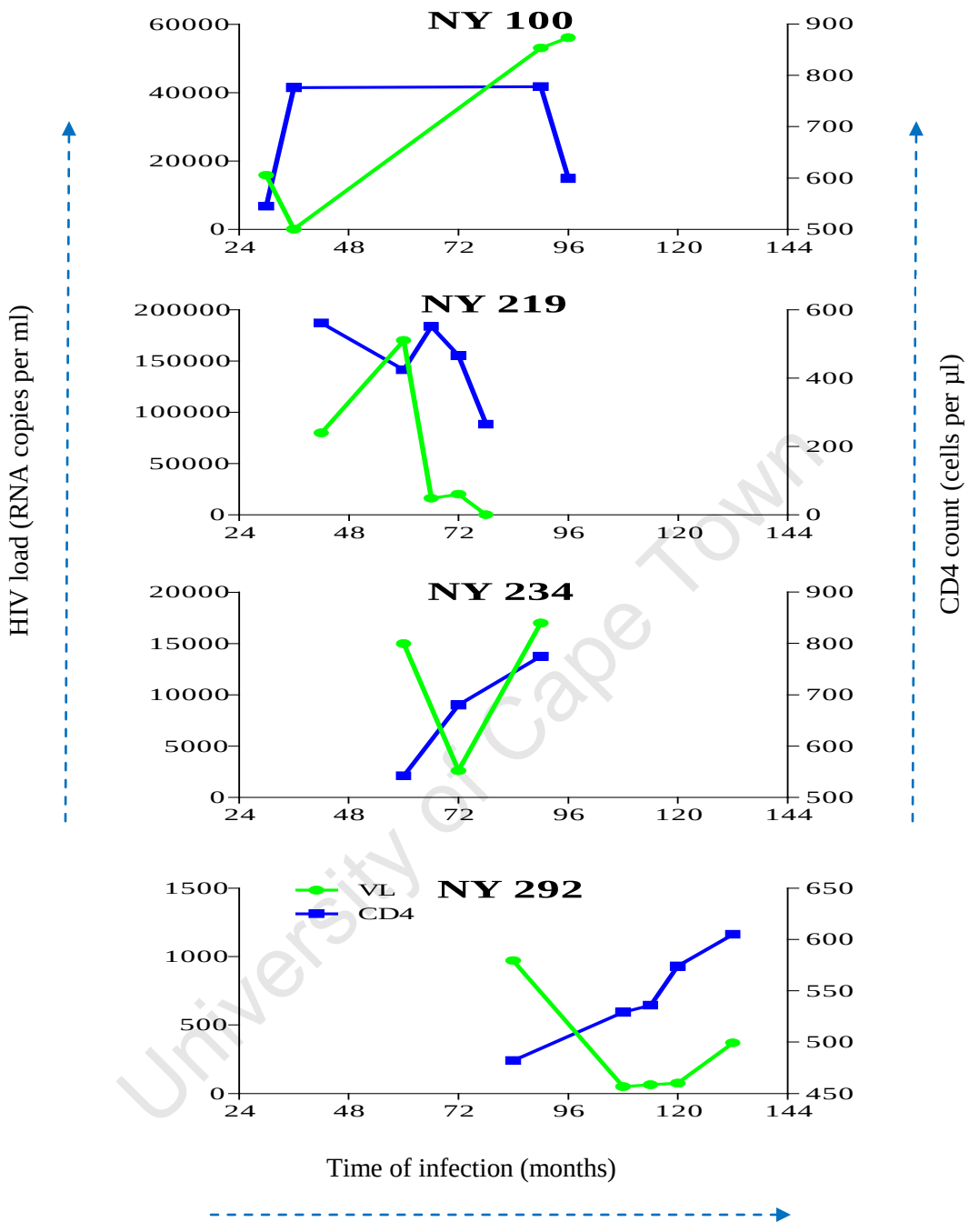
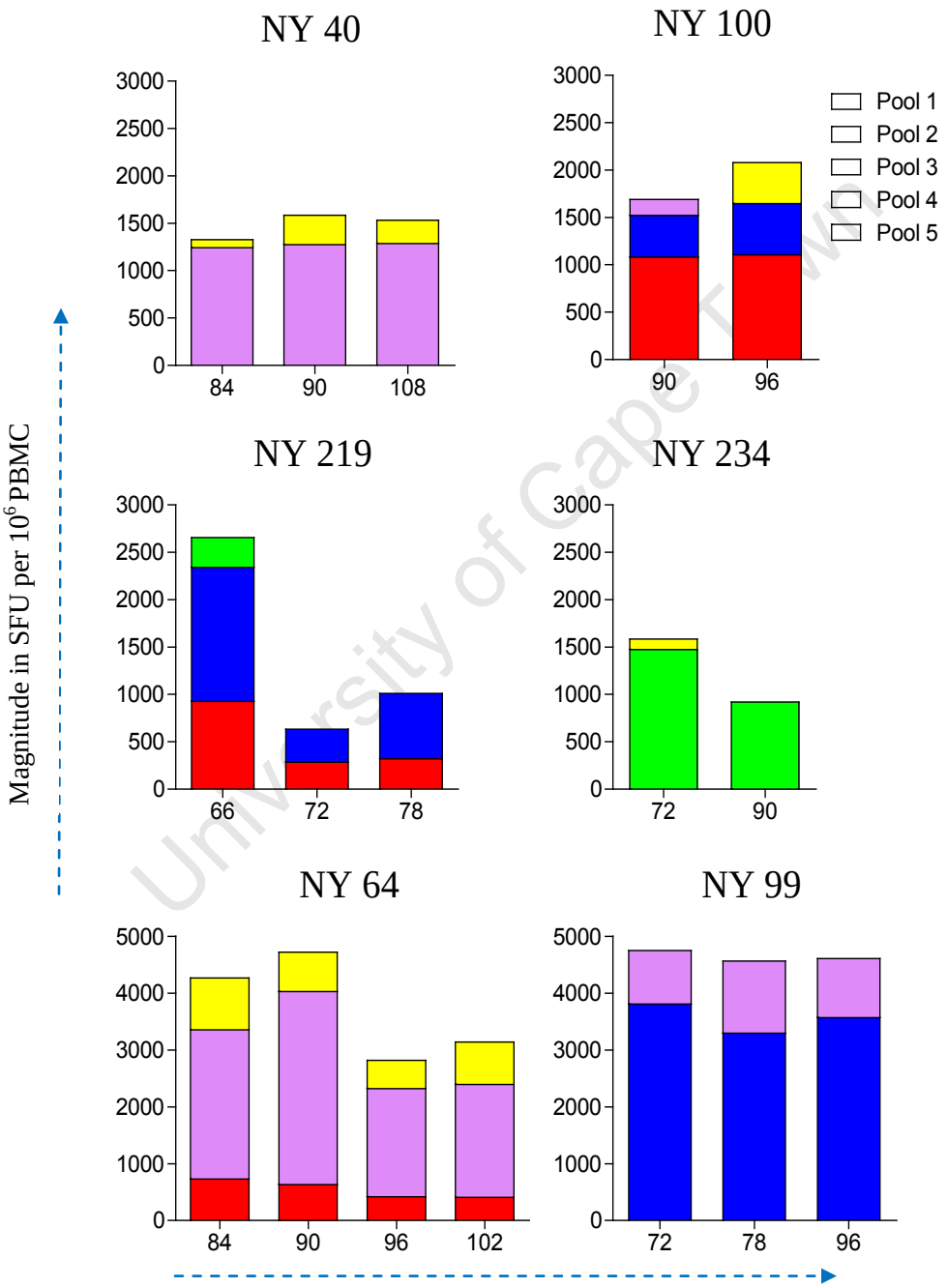


Figure 3.9 Kinetics of CD4 T cell counts (cells/µl) and HIV viral load (RNA copies/ml) in LTNPs (NY010, NY040, NY064, NY094, NY099, NY100, NY219, NY234, NY094 and NY292) who have been chronically infected with HIV for >5 years. HIV viral load is indicated by the green dots and CD4 counts are indicated by blue squares. Participants were followed 6 monthly and CD4 count measurements were taken at every visit.

3.6 Characterization of HIV-specific T cell responses in LTNPs over time

Nine LTNPs women who had been infected for >5 years (without initiation of HAART) were followed longitudinally to determine the fluidity of T cell responses to HIV Gag peptides during chronic HIV infection at 6 monthly intervals (Figures 3.10).



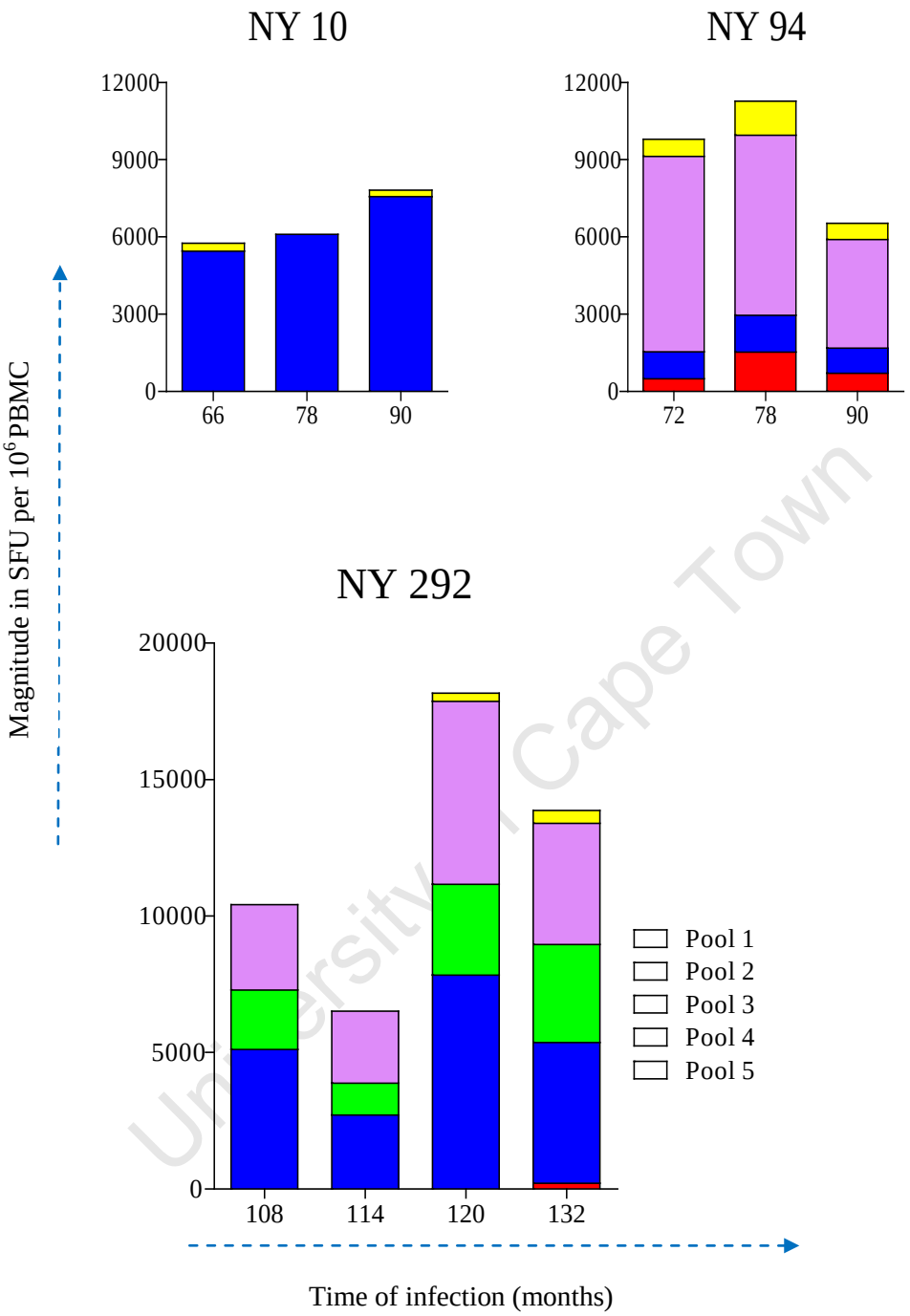


Figure 3.10 Longitudinal evaluations of HIV-1 specific T cell responses to Gag Du422 peptides in HIV-infected LTNPs (NY040, NY100, NY 219, NY234, NY064, NY 099, NY010, NY094, and NY292). Gag pool 1 (red bars) represent peptides contained in p17 of Gag; pool 2 (blue bars), pool 3 (green bars) and pool 4 (purple bars) contained peptides in p24 of Gag; while pool 5 (yellow bars) contained peptides in p15 regions of the HIV-1 Gag protein.

Generally, the magnitude of Gag-specific T cell responses in these HIV LTNPs was higher than those detected in women who were acutely infected with HIV. Furthermore, the Gag pools targeted and the magnitude of responses to each pool was less variable in these HIV LTNPs over time than in acutely infected women. NY292 had the highest T cell responses to Gag (ranging from 6520 to 18160 SFU/10^6 cells). NY040 had the lowest T cell responses to Gag (ranging from 1326 to 1586 SFU/10^6 cells). Although these HIV LTNPs tended to have less variation in their overall magnitude of Gag-responses over time than women during acute infection, the response magnitude and pools targeted remained relatively constant in 2/9 women (NY040 and NY099), declined over time in 4/9 women (NY064, NY094, NY234 and NY219) and increased with time in 3/9 women (NY010, NY100 and NY292).

Variance scores were calculated in the 5/9 long-term chronically HIV-infected individuals who had at least three visits available for analysis. In contrast to the acutely HIV-infected women (Figure 3.6), Figure 3.11 shows that all 5 of these LTNPs had variance scores close to 1 over 18 months of follow-up showing how conserved responses were in these individuals.

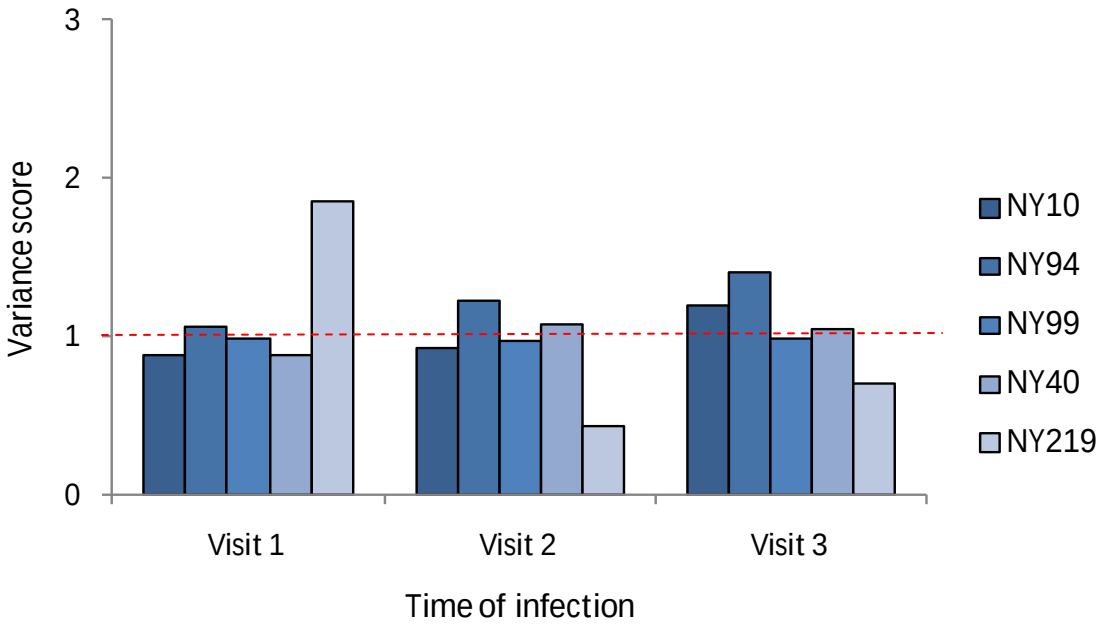


Figure 3.11 Variance scores of five HIV-infected LTNPs. These five women (NY010, NY094, NY099, NY040 and NY219) all had three longitudinal time points available for analysis. The variance scores were determined by dividing the magnitude of Gag (SFU/10^6 cells) generated at a particular time point by the average magnitude at three time points tested. A score approaching 1 means low deviation.

3.7 Contribution of Gag and Nef responses to the overall magnitude of HIV-specific T cell responses during acute versus chronic infection

During acute HIV infection, HIV-specific T cell responses targeted the three proteins of Gag in almost equal proportions, with 38% of responses detected against p17, 32% against p24 and 30% against p15 (Figure 3.12A). During early HIV infection, only two regions of Gag were targeted (p24 and p17) with 58% of responses targeting p17 and 42% targeting p24. In the same women during the chronic phase of HIV-1 infection, p24 and p17 emerged as the co-dominant regions being targeted within Gag and these accounted for 44% and 47% of the total Gag-specific T cell responses measured, respectively. Although p24 (the most conserved region of the Gag protein) showed no outright dominance throughout the coursee of infection, it was the only region that steadily increased in the proportion it was targeted [accounting for 32% of responses during acute infection, 42% of responses during early infection, and 44% of responses during chronic infection). In contrast, p15 was the most variably targeted region of Gag [accounting for 30% of responses during acute infection, none of the responses during early infection, and 9% of responses during chronic infection]. In this study, the p24 region of Gag was made up by Gag peptide pool 2, pool 3 and pool 4. Within these three Gag p24 peptide pools, the end of p24 (pool 4) accounted for the largest proportion of responses (32%) in acutely HIV-infected women and remained the most targeted of p24 regions throughout the course of HIV infection in these women.

In Nef, T cell responses targeting the central most conserved region of this protein (made up of pool 2, pool 3 and pool 4) consistently were the most targeted throughout the course of HIV-1 infection (Figure 3.12B). Nef pools 2 and 4 contributed 13% and 87% respectively during acute HIV infection. During early HIV infection, Nef-specific targeting shifted from pool 4 to pool 2 contributing 53% of the overall Nef responses. During chronic infection in these women, pool 2 emerged as the most dominant Nef pool targeted (accounting for 80% of responses to Nef). Although targeting of the central region of Nef accounted for most of the T cell responses detected against this protein, the exact pools targeted within this central region were highly variable throughout the course of infection.

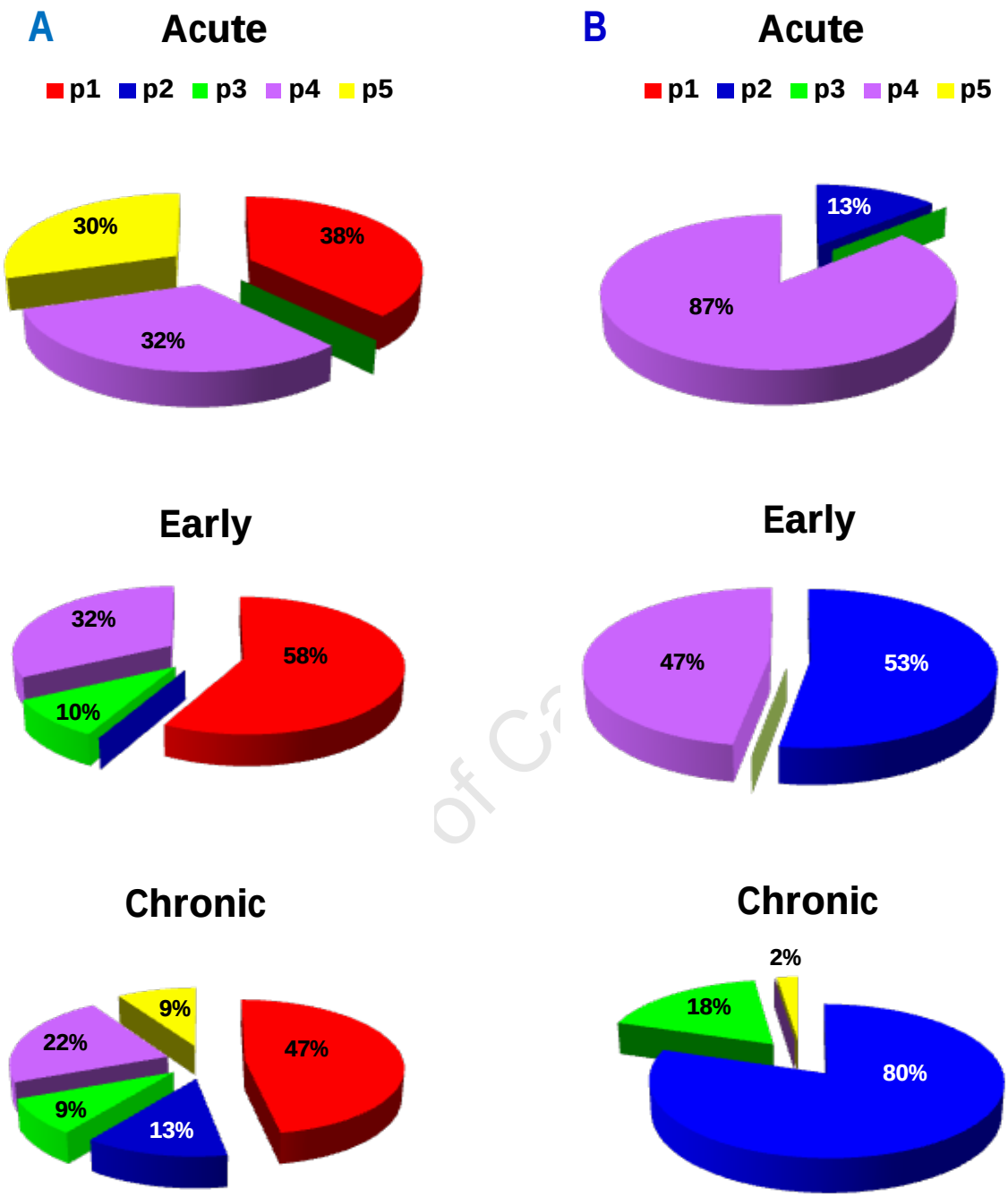


Figure 3.12 Relative contribution of each Gag (A) and Nef (B) peptide pool to the overall magnitude of the T cell responses to each HIV protein over time from acute to chronic HIV infection. The magnitudes are expressed as percentage in seven acutely, 4 early and 3 chronic chronically infected women. (A) Red slices represent Gag p17. Blue, green and purple slices represent pools 2, 3 and 4 of Gag p24. Yellow slices represent p15 of Gag. (B) Blue, green and purple slices represent pools 2, 3 and 4 of Nef (central) and the yellow slice represents the C-Terminal region of Nef.

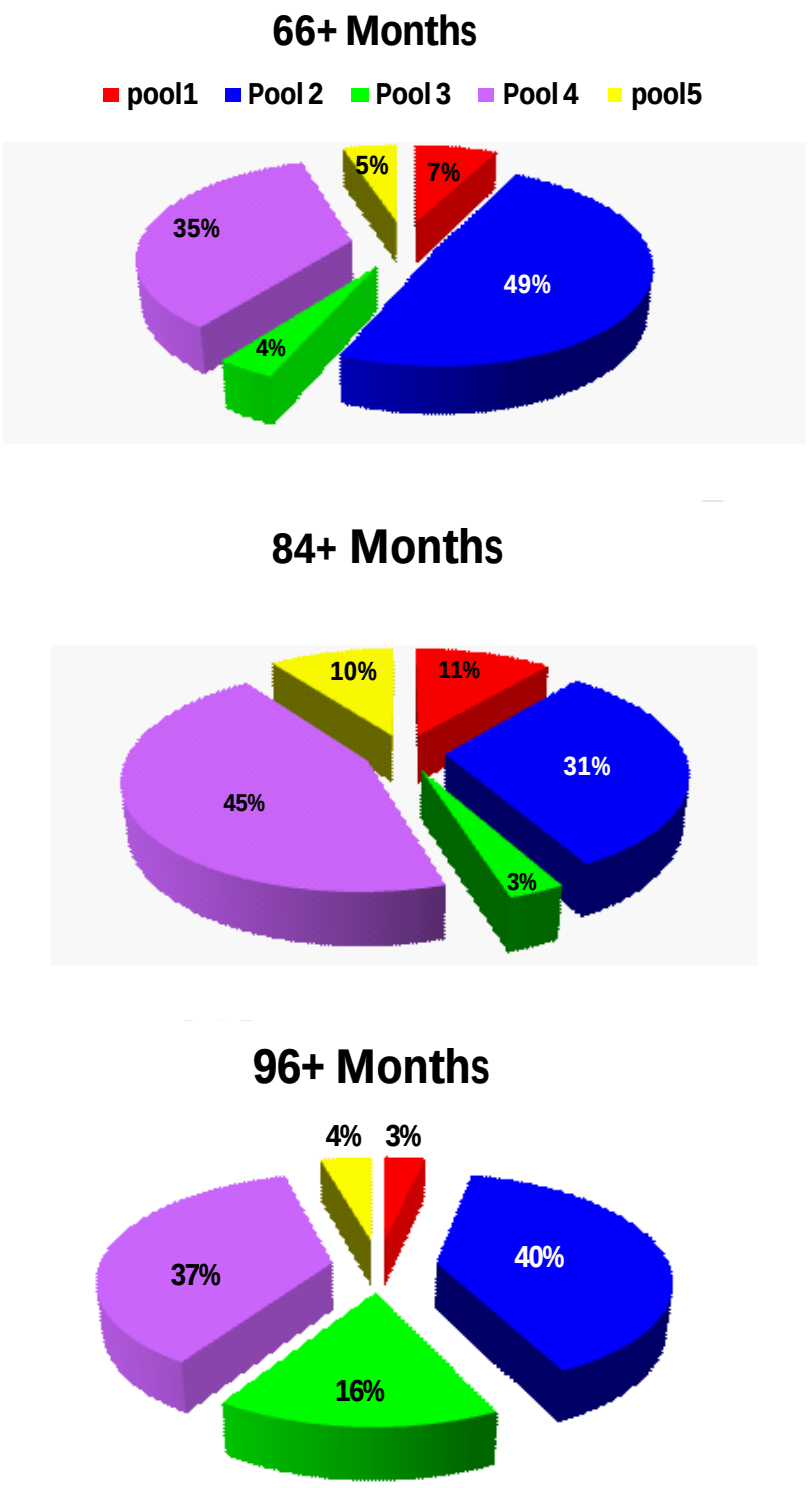


Figure 3.13 Relative contribution of each Gag pool to the overall magnitude of the T cell responses over time in nine LTNPs. Red slices represent Gag p17. Blue, green and purple slices represent pools 2, 3 and 4 of Gag p24, and yellow slices represent Gag p15. The first time point is at 66^{+} months, the second, 84^{+} months and the last 96^{+} post infection.

In HIV-infected LTNPs, T cell responses to p24 contributed 88% of all Gag responses at 66 months of infection, 79% at 84 months and 92% at 96 months post-infection (Figure 3.13). In contrast, responses to p15 and p17 of Gag made up less than a quarter of total Gag responses throughout the course of infection. When the proportionate contribution of each Gag region targeted by T cells (Figure 3.12 and Figure 3.13) wasadjusted for the number of amino acids in each region of Gag (by dividing the magnitude of T cell response by the number of amino acid within that protein to compensate for the fact that p24 was so much longer than p15 or p17), there were slight differences between before and after normalization in LTNPs, although p24 still remained the highest contributor to the overall Gag specific response (Figure 3.14).

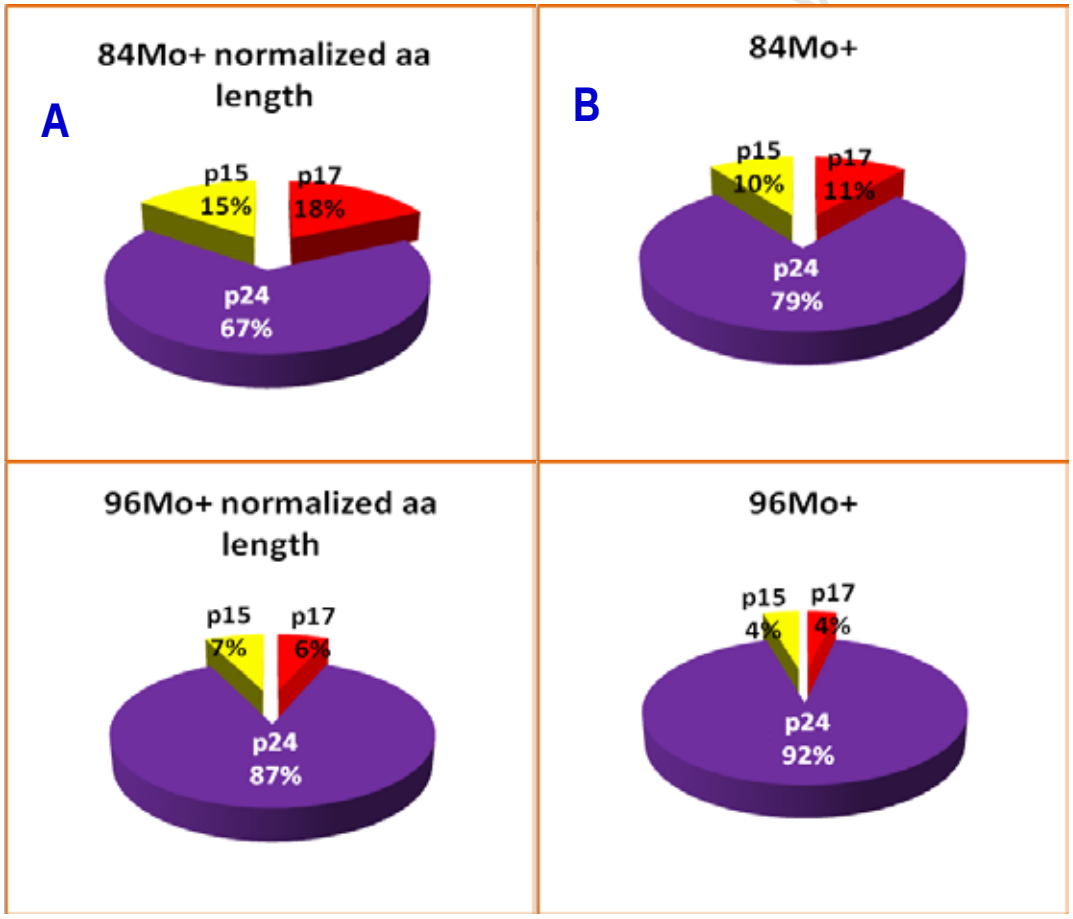


Figure 3.14 Adjustment of Gag-specific T cell responses to compensate for varying lengths of Gag regions in nine HIV-infected LTNPs. The comparisons between normalized (column A) and non-normalized (column B) Gag specific T cell responses to the overall magnitude at 84 months+ and 96 months+ post infection. The magnitude of response to each protein was divided by the number of amino acids within that protein. The yellow slices represent p15, red p17 and purple p24.

3.8 Identification of specific Gag and Nef peptides targeted by HIV-specific T cell responses during the natural course of HIV infection

Individual peptide mapping was carried out longitudinally in 3/7 acutely HIV-infected women (C001, C004 and C008) to investigate how specific HIV regions were being targeted evolved during the course of infection. C001 had returned for eight clinic visits while C004 and C008 had returned for six clinic visits each. Initially, only a few Gag and Nef peptides were recognized during acute HIV infection indicating the breadth of responses was initially narrow but the number of peptides recognized increased over time of infection (Figures 3.15). A total of 94 distinct peptides were identified in Gag and Nef. Of these, 23/94 peptides (24%) were recognized during acute infection, 30/94 peptides (32%) were recognized during early infection, and 41/94 peptides (44%) were recognized during chronic infection. Participant C001 recognized the greatest number of these 94 peptides (46/94 individual peptides), followed by C004 (23/94 individual peptides), and C008 recognized 19/94 peptides. The number of peptides targeted varied at different time points in all three participants; with C001 recognizing the most peptides at month 24 (14 peptides being recognized), C004 peaked at month 12 (7 peptides being recognized) and C008 peaked at month 6 (6 peptides being recognized).

In some women, IFN-γ responses to the individual peptides were conserved longitudinally (Figure 3.15). The most conserved peptide recognized in C001 was Gag 11 with 6/8 time points targeting this peptide (Figure 3.15A). The most conserved peptide recognized in C004 was Nef 19 with samples from 6/6 time points targeting this peptide (Figure 3.15B). In C008, both Gag peptide 19 and Nef peptide 19 were recognized simultaneously at 4/6 time points (Figure 3.15C). In contrast, recognition of other peptides was more fluid. In C001, Nef 21 was only recognized during chronic infection and not during acute infection. In C008, Gag peptides 74 and 88 were only recognized during acute and early infection respectively and lost during chronic HIV infection.

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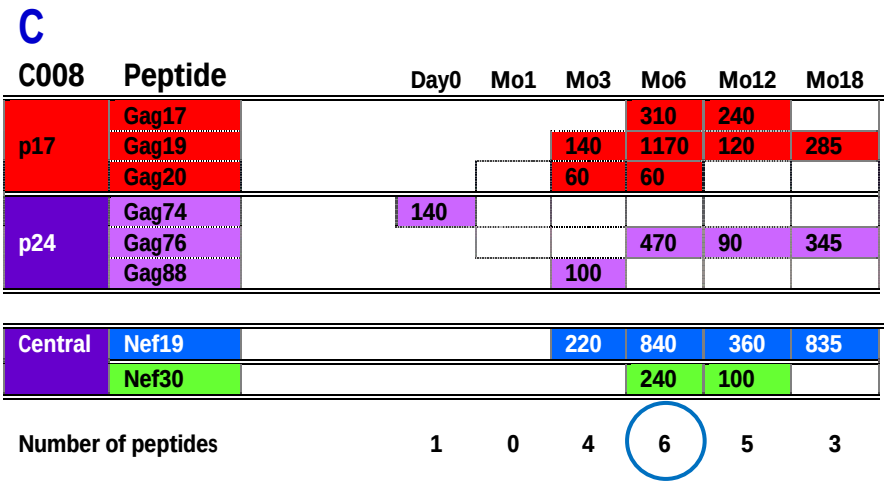
A

C001	Peptide	Day0	Mo1	Mo3	Mo6	Mo12	Mo18	Mo24	Mo30
p17	Gag3			60	960	600	70		
	Gag4							140	
	Gag9						50		
	Gag11	110*		130	60	320		90	120
	Gag17							60	56
	Gag20					60	270	140	60
p24	Gag35						50		180
	Gag47							80	
	Gag60		57*					60	
	Gag66						70	120	
	Gag69							60	
	Gag76								420
	Gag80			50	50			270	
p15	Gag97						50		
	Gag99	90*				230	120	150	150
Central	Nef11							90	
	Nef20				140		170	340	600
	Nef21						300	340	850
	Nef30							140	
C-terminal	Nef47								160
Number of peptides		2	1	3	4	4	9	14	9

B

COO4	Peptide	Day0	Mo3	Mo6	Mo12	Mo18	Mo24
p17	Gag5		230	60	50		
	Gag6			405			
	Gag13				180		
	Gag16		60			150	
p24	Gag34		50				
	Gag35				100		
	Gag38		60	130	260		
	Gag54				180		
	Gag62				120		
	Gag64					130	130
Central	Nef16						110
	Nef19	410	260	180	100	650	1390
Number of peptides		1	5	4	7	3	3

The squares marked with an * is when the peptide was not confirmed and the magnitude of the pool response was substituted



Figures 3.15 Mapping of HIV-specific Gag and Nef peptides targeted longitudinally in three women over a period of 18-30 months following acute infection. The HIV-1 proteins are Gag p17 (red), p24 (dark purple) and p15 (yellow) and the Nef protein constitute the central conserved (dark purple) and C terminal regions (yellow). The peptide columns represent the peptide recognized by Nef or Gag specific T cell responses. Each colored square going across represents the breadth of the recognized peptide at a particular time point. The color of the square represents the pool in which the peptide is situated; peptides recognized within Gag or Nef: - pool 1(red); pool 2(blue); pool 3(green); pool 4 (purple) and pool 5 (yellow). The number within the square represents the longitudinal (magnitude of response in SFU per million PBMC) responses of the recognized peptide over time. The greatest number of peptides targeted is circled in blue.

Figure 3.16 summarizes the overall proportion of peptides targeted from acute to chronic HIV infection in Gag and Nef. During acute infection, almost equal numbers of Gag peptides within the p15 and p17 regions were recognized. During early infection there was a shift in peptide recognition with equal peptide clustered in p24 and p17. During the chronic HIV infection, peptides were recognized within all three regions although p15 peptides were less well recognized than both p17 and p24 peptides. The number of peptides recognized within p15 was lowest during early and chronic infection. In Nef, the highest number of peptides recognized was in the central part of Nef and this pattern was maintained throughout the course of infection (Figure 3.16B).

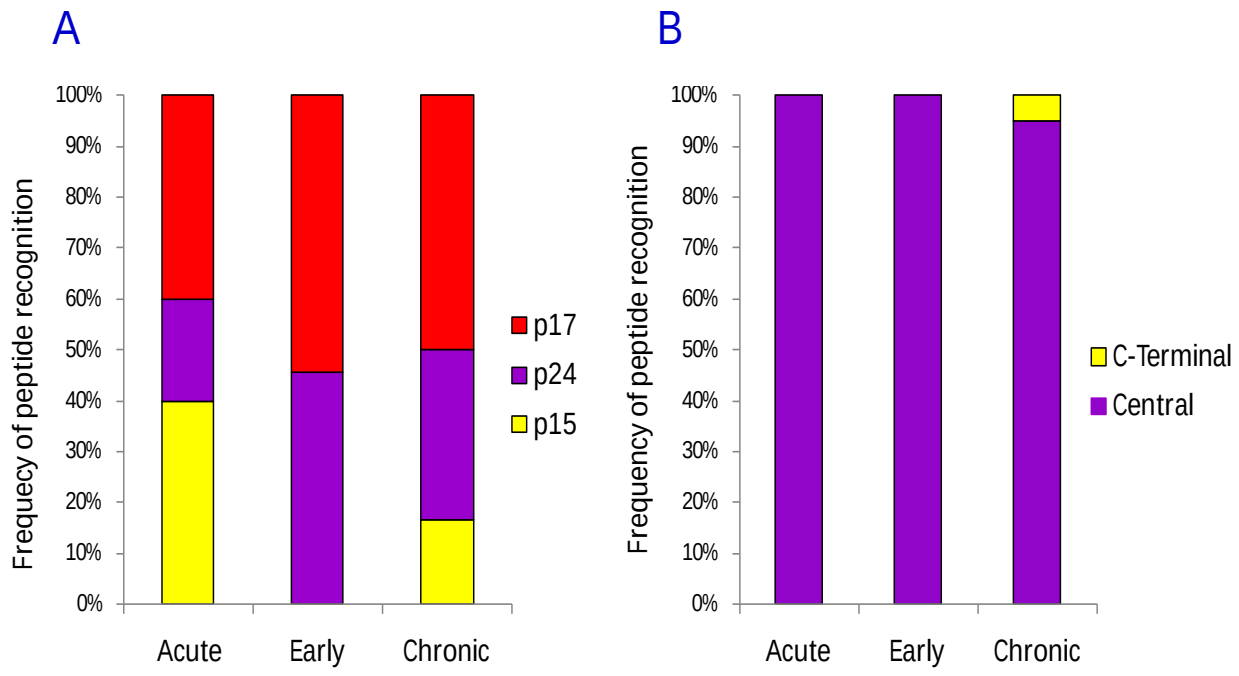


Figure 3.16 Summary of the frequency of peptide recognition within Gag (A) and Nef (B) from acute to the chronic HIV-1 infection. The overall number of peptides recognized is expressed as percentage in seven acutely, 4 early and 3 chronic chronically infected women. The red bars represent the peptides recognized in p17, purple p24 and yellow p15 of Gag. Peptides recognized within Nef; purple bars, central and yellow bars, C- terminal.

The breadth of Gag-specific T cell responses in the 9 LTNPs is shown in Figure 3.17. These LTNPs targeted a total of 121 individual peptides in Gag during follow-up. Of these 121 individual peptides, 20/121 peptides (17%) were from the p17 region of Gag, 82/121 peptides (67%) were from p24, and 19/121 (16%) were within the p15 region of Gag. In these chronically infected LTNPs, the number and identity of each peptide remained relatively constant over time with one exception. While participant NY292 targeted only 5 peptides at 108 months post-infection, the breadth of her responses increased steadily to 13 peptides being recognized at 132 months post-infection. Gag p24 was consistently the most targeted region with 61% of the peptides being recognized in this region at 66 months; 59% of the peptides being recognized at 84 months; and 77% of the peptides being recognized at 96 months post-infection (Figure 3.18).

NY 10	Peptides		Mo66	Mo78	Mo90
p24	Gag35		165		370
	Gag45		3925	5077	6890
p15	Gag93		425		120
Number of peptides			3	1	3

NY 40	Peptides		Mo84	Mo90	Mo108
p24	Gag73		730	960	1370
	Gag74		600	580	900
	Gag83				130
p15	Gag106		80	70	200
Number of Peptides			3	3	4

NY 64	Peptides		Mo84	Mo94	Mo96	Mo102
p17	Gag6		720	660	660	667
p24	Gag73		1770	1810	3160	2677
	Gag83		1430	2250	2140	2567
	Gag87		230	150	120	167
p15	Gag105			140	500	
	Gag106		970			1897
Number of peptides			5	5	5	5

NY94	Peptides		Mo72	Mo78	Mo90
p17	Gag8		153	190	340
	Gag10		153	320	140
	Gag20		183	300	600
p24	Gag36		623	860	1360
	Gag73		313	340	260
	Gag87		2553	1950	4500
	Gag88		3633	3170	5340
p15	Gag106		313	510	
	Gag107		443		700
Number of peptides			9	8	8

NY99	Peptides		Mo72	Mo78	Mo96
p24	Gag34		160	240	895
	Gag40		3807	3320	3815
	Gag73		640	880	1425
Number of peptides			3	3	3

NY 100	Peptides		Mo90	Mo96
p17	Gag8		1217	1300
	Gag14		277	
p24	Gag40		937	860
	Gag74		237	
p15	Gag106			200
Number of peptides			4	3

NY 219	Peptides		Mo66	Mo72	Mo78
p17	Gag20		1235	350	330
p24	Gag36		2035	410	1070
	Gag69		1245		
Number of peptides			3	2	2

NY 234	Peptides		Mo72	Mo90
p24	Gag65		1040	620
p15	Gag105		800	
Number of peptides			2	1

NY 292	Peptides		Mo108	Mo114	Mo120	Mo132
p17	Gag4					210
p24	Gag35		113		667	
	Gag42					120
	Gag44		1523	713	5067	5430
	Gag45		1553	1053	4087	5360
	Gag53					110
	Gag62		513	313	2687	2720
	Gag67			173	667	890
	Gag72		113	135	394	4420
	Gag73			713	3687	4290
	Gag75					150
	Gag83					150
p15	Gag93				137	240
	Gag111				387	480
Number of peptides			5	6	9	13

Figures 3.17 Mapping of HIV-specific Gag peptides targeted longitudinally in nine LTNPs that were studied longitudinally. The peptide columns represent the Gag peptides recognized: Gag p17 (red), p24 (dark purple) and p15 (yellow). Each colored square going across represents the breadth of the recognized peptide at a particular time point. The color of the square represents the pool in which the peptide is situated; peptides recognized within Gag: - pool 1(red); pool 2(blue); pool 3(green); pool 4 (purple) and pool 5 (yellow). The number within the square represents the longitudinal (magnitude of response in SFU per million PBMC) responses of the recognized peptide over time.

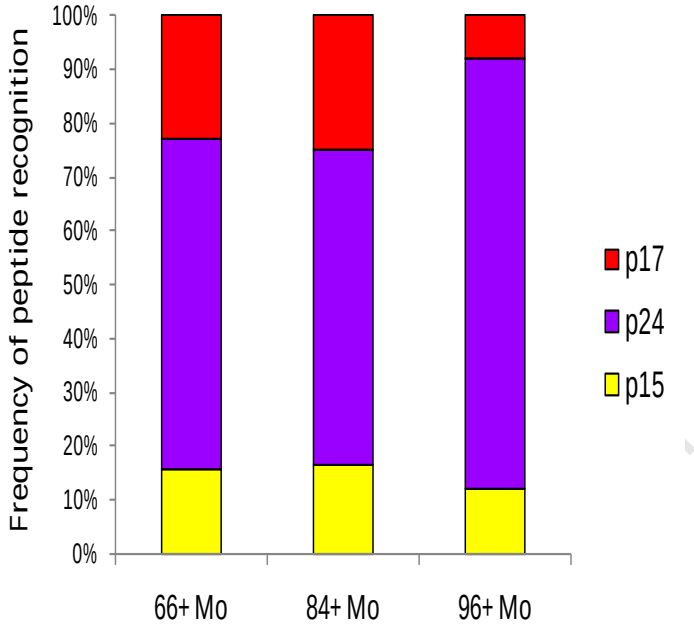
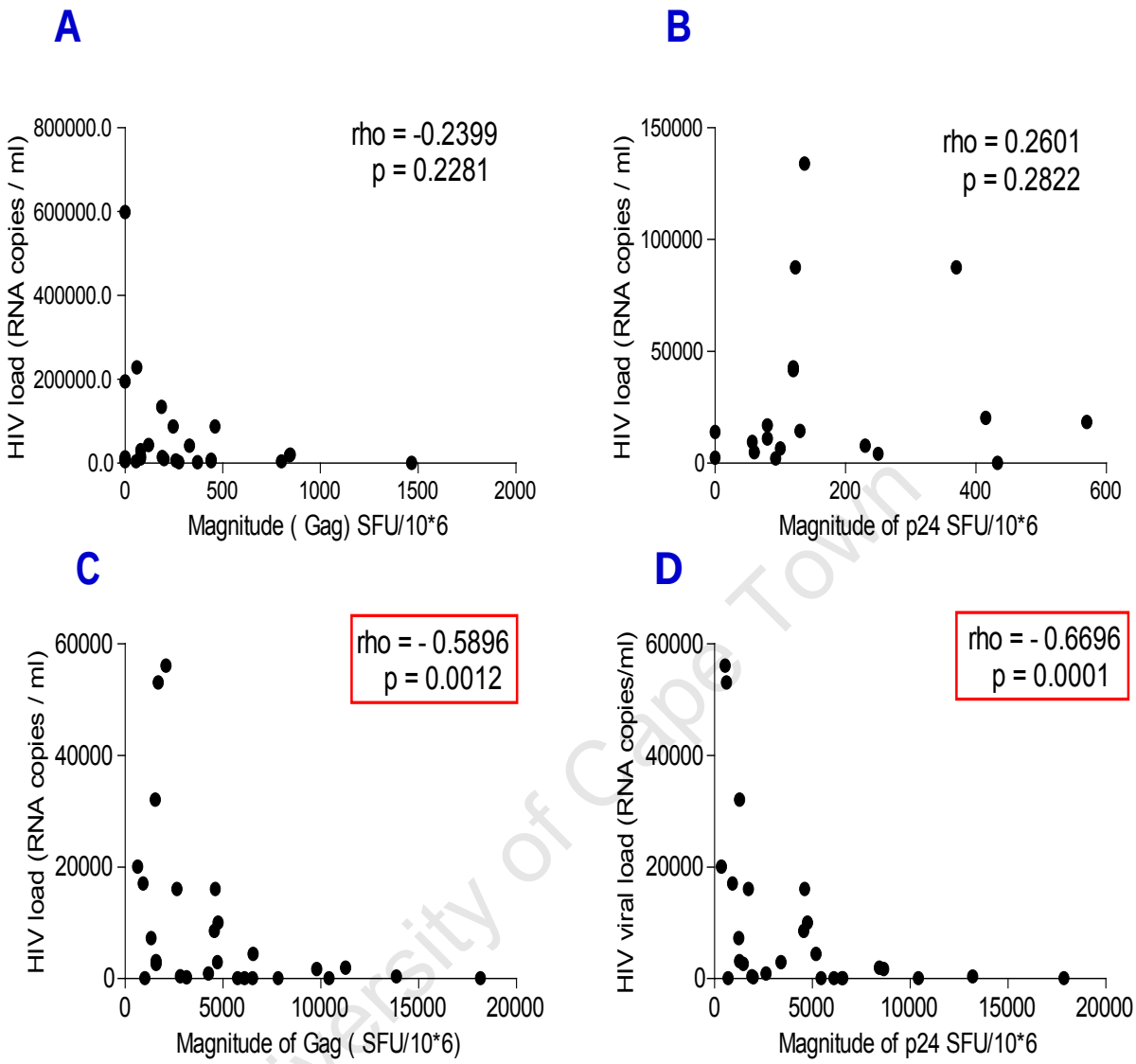


Figure 3.18 Summary of the frequency of peptide recognition within Gag in nine HIV- LTNP infected women. The overall number of peptides recognized is expressed as percentage at 66 months+, 84 months+ and 96 months+ post infection. The red bars represent the peptides recognized in p17, purple p24 and yellow p15 of Gag.

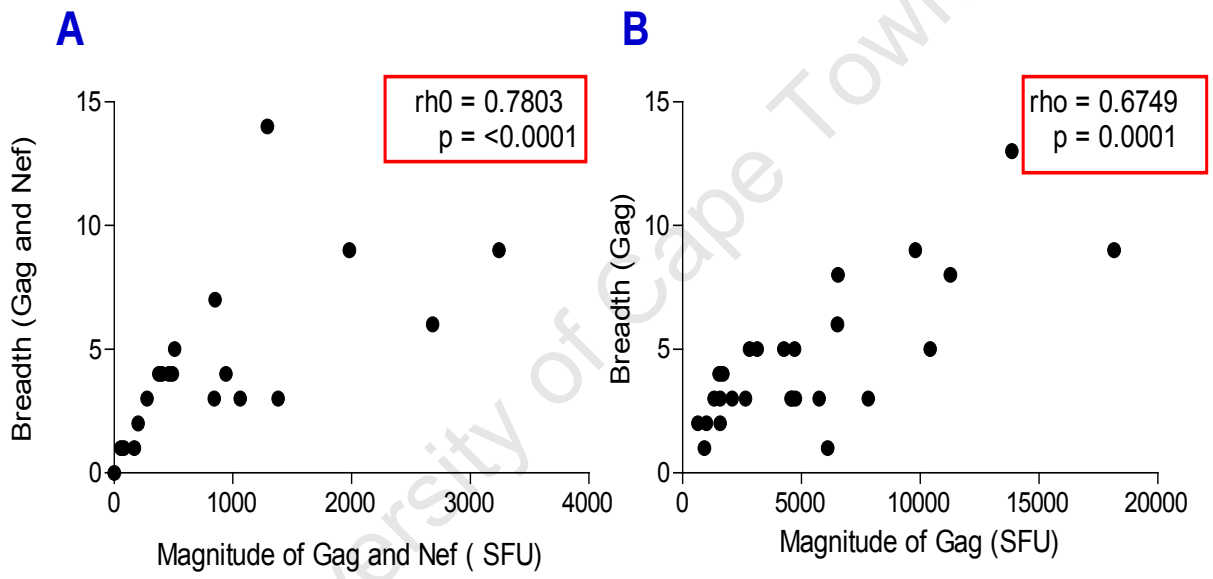
3.9 Relationship between magnitude and breadth of T cell responses and HIV clinical status during acute versus chronic HIV infection

The relationship between HIV clinical status (CD4 T cell counts and plasma viral loads) and the magnitude of T cell responses to Gag peptides was investigated during both acute and chronic HIV infection (Figure 3.19). An inverse relationship was observed between the magnitude of T cell responses to Gag and plasma viral load following acute HIV infection although this was not significant (Figure 3.19A; Rho=-0.24; p=0.2). A significant negative association was found between the overall magnitude of T cell responses to Gag (and specifically the p24 region) and plasma viral load in the 9 LTNPs (Figure 3.19C: Rho=-0.59; p=0.001 for whole of Gag; Figure 3.19D: Rho=-0.67; p=0.0001 for p24).



Figures 3.19 Relationship between plasma viral load and T cell responses to Gag and p24 in women acutely infected with HIV (A-B) compared to LTNPs (C-D). A and B: Each dot represents matched measurements of HIV viral load (RNA copies/ml) and the magnitude (SFU/10^6PBMC) following acute infection. **C and D:** Each dot represents matched measurements of HIV viral load (RNA copies/ml) and the magnitude (SFU/106 PBMC) during long-term chronic infection. Spearman correlations were used and p values < 0.05 were considered as significant.

When the impact of the HIV clinical status on the breadth of T cell responses to Gag and Nef was assessed, no correlation was observed between the breadth of responses to Gag or Nef and plasma viral load in both the acute and LTNP cohorts (Rho=-0.11; p=0.64 for acute women; Rho=-0.29; p=0.14 for long-term chronic women; results not shown). Although I did not observe an association between breadth of responses and clinical status, a significant positive correlation was found between the overall magnitude of T cell responses to Gag and/or Nef and the breadth of responses in both acutely (Rho=0.78; p<0.0001) and LTNP infected women (Rho = 0.68; p=0.0001; Figures 3.20).



Figures 3.20 Relationship between overall magnitude of T cell responses to Gag or Nef and the number of peptides being recognized in acutely infected women and LTNPs. **A**: Each dot represents the matched measurements of magnitude (SFU/10^6 PBMC) and breadth of the total HIV-Gag and Nef specific T cell responses following acute infection. **B**: Each dot represents the matched measurements of magnitude (SFU/10^6 PBMC) and breadth of the total HIV-Gag specific T cell responses in LTNP infected women. Spearman correlations were used and p values < 0.05 were considered as significant.

3.10 Relationship between immunodominant responses to Gag or Nef in the control of HIV infection

Table 3.6 summarizes the individual peptides targeted during acute HIV infection in the three women who were followed longitudinally that were characterized in this study as being immunodominant. Immunodominance was determined based on the fact that these individual peptides yielded the highest magnitude responses by ELISPOT in each woman. During the acute phase of infection in participant C001, Gag 11 [LERFALNPGLLETSE] from p17 was the immunodominant peptide recognized and remained immunodominant during early infection. In participant C008, Gag 74 [YVDRFFKTLRAEQAT] was identified as the immunodominant peptide response. In participants C004 and C008, Nef 19 [QVPLRPMTYKAAFDL] from the central conserved region of Nef was the immunodominant peptide recognized. In participant C004, this Nef peptide remained the immunodominant peptide recognized in early infection. During chronic HIV infection, there was a shift in the immunodominance patterns in two participants (C001 and C008). The immunodominant peptide recognized in participant C001 shifted from Gag 11 [LERFALNPGLLETSE] recognized during acute infection to Gag 3 [RGEKLDKWEKIRLRP] during the chronic phase of infection, although these peptides were both within the p17 region of Gag. The only peptide targeted in acute infection in C008, was Gag 74. The response then shifted to Nef 19 [QVPLRPMTYKAAFDL] and Gag 19 [EELKSLYNTVATLYC] in early and chronic infection.

Generally, the immunodominance patterns in LTNPs was maintained longitudinally with 89% of the peptides recognized within the p24 region and 11% of the peptides recognized in the p17 region (Table 3.6). Participant NY100 recognized peptide Gag 8 [YMLKHIVWASRELER] that is situated in the p17 region of Gag while the remaining participants targeted the p24 region. NY010 and NY292 both recognized Gag 45 [TPQDLNTMLNTVGGH] while NY040 and NY064 recognized Gag 73 [GPKEPFRDYVDRFFK]. NY219 recognized peptide Gag 36 [HQAISPRTLNAWVKV], NY099 recognized Gag 40 [EEKAFSPEVIPMFTA], NY234 recognized Gag 65 [DIYKRWIILGLNKIV], and NY094 recognized Gag 88 [GVGGPGHKARVLAEA].

Table 3.6 Immunodominant epitope and HLA predictions.

PID	Phase	Peptide	Region	Sequence	HLA ClassI
C001	Acute	Gag11	p17	L**ERFAVNPGL**LETSE	B*27
				LE**RFAVNPGLL**ETSE	B*63
C004		Nef 19	Central	**QVPLRPMTYK**AAFDL	B*27,A*11,A*3,A*35
				Q**VPLRPMTY**KAAFDL	B*35,B*42
C008		Gag 74	p24	**YVDRFFKTL**RAEQAT	B*15.A*26,B*70
C001	Early	Gag11	p17		
C004; C008		Nef 19	Central		
C001	Chronic	Gag 3	p17	**SGGEKLDRWEKIRLRP**	B*44,B*60
				R**GELDRWEKI**RLRP	B*4002; B*40
				RGE**ELDRWEKIRL**RP	B*63
C004		Nef 19	Central		
C008		Gag 19	p17	EELK**SLYNTVATL**YC	A*02
NY100	LTNP	Gag8	p17	YMLKH**IVWASRELER**	A*11
NY219		Gag36	p24	HQA**ISPRTLNAW**VKV	B*57;B*5801
NY99		Gag40	p24	**EEKAFSPEV**IPMFTA	B*4501
				EE**KAFSPEVI**PMFTA	B*57
				EE**KAFSPEVIPMF**TA	B*57;B*5801
NY10; NY292		Gag45	p24	**TPQDLNTML**NTVGGH	B*0702;B*4201;
NY234		Gag65	p24	**DIYKRWII**LGLNKIV	B*0801
				DIY**KRWIILGLNK**IV	B*27
NY40; NY64		Gag73	p24	P**FRDYVDRFF**KTLRA	Cw *18
				PFR**DYVDRFFKTLR**A	A*24
NY94		Gag88	p24	GVG**GPGHKARVLA**EA	B*7

The underlined portion of the peptide represents the previously defined epitope as described in the Los Alamos database (http://www.hiv.lanl.gov/content/hiv-db/ELF/epitope_analyzer.html; accessed 10 January 2011).

The HLA typing of the women in this study was not available. However, some of these immunodominant peptides recognized contained well-characterized epitopes (Table 3.6). Participant C004 recognized peptide Nef 19 which contains the epitope VPLRPMTY (VY8). VY8 has previously been reported to be an HLA-B*35 restricted epitope and it is present in about 20% South Africans with HIV-1 subtype C infection (Kiepiela *et al.*, 2004). VY8 was also found to be predominantly targeted during acute infection (Streeck and Nixon, 2010), which was consistent with

the finding of this study. Participant C008 recognized peptide Gag 19 in p17 that contains the 9 amino acid epitope SLYNTVATL (SL9). SL9 has previously been defined as an A*02 restrictive epitope and the likely hood of being targeted by specific T cells during chronic infection is 20% higher than being targeted during acute infection (Goulder *et al.*, 2001; Streeck and Nixon, 2010). NY234, a LTNP, recognized peptide Gag 65 in p24 that contains the epitope KRWIILGLNK (KK10). KK10 is a well known HLA-B*27 restricted epitope and was found to be a rare HLA allele expressed in South Africans with HIV-1 subtype C infection (Kiepiela *et al.*, 2004). T cells recognizing KK10 are more commonly detected during acute infection and this specificity is associated with immune control (Streeck and Nixon, 2010). In this study, however, it was found to be an immunodominant epitope in an HIV-infected LTNP.

CHAPTER 4

Discussion

CHAPTER 4

Discussion

More than a decade has passed since the first studies described the temporal association between HIV-1 specific T cell responses and decreases in viremia during acute infection, suggesting the crucial role for T cells in the initial control of viral replication (Koup *et al*., 1994; Borrow *et al*., 1994; Schmitz *et al*., 1999; Jin *et al*., 1999). Subsequent studies have shown that CTLs can inhibit viral replication in LTNPs (Betts *et al.*, 2006) and that effective pressure exerted by CTLs can cause HIV to rapidly escape by mutation during acute (Goonetilleke *et al*., 2009) and chronic infection (Draenert *et al*., 2004). In South Africa, HIV-1 subtype C is the predominant subtype causing infection. Knowledge of T cell responses during acute infection and their role in HIV-1 subtype C pathogenesis will advance our understanding of the first immune responses behind immune control. The aim of this study was to longitudinally map HIV-1 subtype C Gag and Nef-specific T cell responses by ELISPOT in recently infected women (mean 21 days ± 13) and compare these to LTNPs who had been infected for more than 5 years. T cell responses to HIV Du422 Gag and Nef peptides were mapped at the single peptide level. T cell responses to Gag and Nef during acute and chronic HIV infection were compared with markers of disease progression (plasma CD4 counts and HIV RNA load).

Evolution of HIV-specific responses during acute infection and disease control

During acute HIV infection, the breadth of HIV-specific T cell responses to both Gag and Nef peptide pools was narrower and responses were lower in magnitude compared to responses detected in the same women during chronic infection. More women had detectable HIV Gag responses during acute infection than Nef although responses to Nef were generally higher in magnitude. The kinetics of these HIV Gag and Nef specific T cell responses during acute infection and immunodominant peptides recognized did not remain static over time but fluctuated during the first months of infection. Although T cell specificities and response magnitudes during acute HIV infection were fluid as previously described by Mlotshwa *et al*. (2010), the magnitude of HIV-specific responses were significantly associated with the breadth of responses.

In women who were acutely infected with HIV, blood CD4 T cell counts were inversely associated with plasma HIV-1 RNA loads confirming that women with higher viral loads experienced greater CD4 T cell loss. During acute infection, studies have shown that HIV

replicates rapidly and spreads throughout the body especially in the GALT, which are densely populated with CD4 CCR5 memory T cells (Brenchley *et al*., 2004; Picker *et al*., 2006). Approximately 80% of these CD4 T cells are depleted following 3 weeks of infection (Brenchley *et al*., 2004). In blood, there is an exponential increase in viremia during acute HIV infection which reaches a peak of ≥ 1 million RNA copies per ml which corresponds with a low CD4 count at the time of the peak (Fiebig *et al*., 2003). This is followed by a slower rate CD4 decline from about five to six months (Schacker *et al*., 1997). In untreated individuals, viremia plateaus at about 6 -12 months and can remain stable for several months or years until progressive CD4 loss results in the onset of AIDS (Centlivre *et al*., 2007). Consistent with this study, other studies have shown that during primary HIV subtype C infection, the mean plasma RNA inversely correlated with mean CD4 count (Gray *et al*., 2005; Novitsky *et al*., 2009).

Mlotshwa *et al*. (2010) recently investigated the pattern of HIV-specific T cell responses during acute and early infection in 53 individuals by IFNγ ELISPOT. As early as 2-8 weeks post infection, 75% of acutely infected individuals recognized epitopes in Nef while 50% of individuals recognized epitopes in Gag. Although this study included far fewer individuals, only 27% of women recognized Nef during acute infection while 57% of women recognized Gag. At 6 months post infection, Mlotshwa *et al*. (2010) found 100% of individuals targeted Nef and 60% of individuals targeted Gag. In this study, all women targeted Gag and Nef by 6 months post infection. Longitudinally, Mlotshwa *et al*. (2010) found that responses against Nef were the highest in magnitude compared with any other HIV protein and this study also found that Nef responses were generally of higher magnitude than responses against Gag. Quality control T cell responses to CEF were measured in each experiment and included on each ELISPOT plate to control for plate-to-plate and assay-to-assay variation that would impact on the interpretation of the results from this study. In the QC samples, CEF responses were found to fall within the established CV ranges with minimal fluctuation from the mean (Appendix 1 Figure A3). This suggests that the longitudinal fluctuations observed in the magnitude of Gag and Nef specific T cells in this study were not due to plate or assay variation but reflected accurate trends in Gag and Nef recognition for each woman.

Turnbull *et al*. (2009) analyzed longitudinal HIV-specific T cell responses in 21 acutely HIV-infected individuals and showed that the HIV epitopes recognized were generally narrow during acute infection and broadened with disease progression which is in agreement with this and other studies (Altfeld *et al*., 2001; Lichterfeld *et al*., 2004; Gray *et al*., 2009; Mlotshwa *et al*., 2010). Similar to the findings presented in this study, Turnbull *et al.* (2009) demonstrated that the HIV epitopes recognized fluctuated over time, resulting in hierarchical shifts.

Of the three acutely infected HIV women studied to chronic stages of infection, C001 have shown the slowest rate of disease progression with a mean viral load of 4860 RNA copies/ml from month 1 to month 18. At month 24, however, her viral load increased four-fold to 20200 RNA copies /ml. At month 3, her only T cell response was directed at Gag 60 [TSTLQEQIAWMTSNP] which is situated in p24 of Gag which contains the HLA-B57/58 TW10 restricted epitope which is associated with slow progression (Goulder and Watkins, 2008). After 3 months, her response to this peptide [TSTLQEQIAWMTSNP] was lost although it did re-emerge at 18 months of infection. While she lost this response relatively early, several studies have shown that a common escape mutation induced by this response is the T242N in [TSTLQEQIAWMTSNP] which is associated with a less fit virus (Kiepiela *et al*., 2007; Goulder and Watkins, 2008). At month 24, despite recognizing the TW10 epitope [TSTLQEQIAWMTSNP] again, she failed to continue controlling her viral load and this could possibly be due to an increase in the breadth of her response to Nef, which may have dampened her ability to control viral replication. Several studies have shown that targeting of Nef and Env is associated with increased viral loads (Masemola *et al.*, 2004; Zuniga *et al.*, 2006; Turnbull *et al*., 2009; Ferre *et al.*, 2010) and this could account for the lack of viremic control in C004 and COO8 where Nef was predominantly targeted.

Goonetilleke *et al*. (2009) showed that the first emerging T cell responses during acute infection rapidly select escape mutations whichcoincided with decline from peak viremia and it is likely that the loss and emergence of T cell responses to HIV proteins reported in this study may similarly reflect escape of HIV from T cell pressure exerted by immunodominant responses which are replaced by the next most dominant T cell response.

HIV-specific T cell responses in LTNP are comparatively stable over time

In the nine LTNPs (infected for >5 years), the kinetics of Gag-specific T cell responses were generally higher, more stable and frequency of epitopic recognition were more conserved than measured in the acutely HIV-infected women. IFN-γ responses in these individuals preferentially targeted the highly conserved p24 region of Gag. While the magnitude of T cell responses to Gag (particularly the p24 region of Gag) in LTNPs was significantly inversely associated with plasma viral loads at the same time point; no similar correlation was observed in women who were acutely infected with HIV. Generally, no association was observed between the breadth of HIV-specific T cell responses and plasma RNA viral loads in either acutely infected women or LTNPs. Several studies have shown that targeting of Gag by HIV-specific T cells was protective. In a previous study with a large cohort of 578 chronically infected participants, it was shown that individuals who targeted Gag controlled vireamia better than individuals who targeted Env (Kiepiela *et al*., 2007). Masemola *et al.* (2004) showed, in a mixed cohort of recently and chronically infected individuals, that preferential targeting of Gag was associated with low viral load while targeting of Nef was associated with high viral load. Another study of recently infected individuals has shown that HIV-specific T cells targeting p24 region of Gag were associated with immune control (Streeck *et al*., 2007).

In contrast, no correlation was found between CD4 T cell counts and plasma viral loads in LTNPs suggesting that women who are controlling viraemia and maintaining CD4 counts do not exhibit the same relationship between viraemia and CD4 loss as those progressing normally. Cao *et al*. (1995) previously reported that only about 5% of untreated chronically infected individuals with stable CD4 counts above 500 cells /μl (LTNPs) are able to immunologically control infection for longer than 10 years. A small subset (<1%) of these individuals, known as elite controllers, maintain a viral load of less than 50 RNA copies/ml (Madec *et al*., 2005; O'Connell *et al.*, 2009) suggesting very slow or undetectable disease progression in these individuals. This is in contrast to rapid progressors who eventually succumb to AIDS within 3-5 years of infection (Poropatich and Sullivan, 2011). A small percentage of LTNP (±7%) may eventually progress to AIDS due to opportunistic infections. Similarly, about 7% of elite controllers may meet the clinical definition of AIDS, where individuals with less than 75 copies per ml had CD4 T cell counts of less than 350 cells /μl, suggesting that viral loads of elite

controllers did not correlate with maintenance of CD4 counts in blood (Poropatich and Sullivan, 2011).

All nine LTNPs in this study predominantly responded to Gag throughout the time points included in this study and generally maintained CD4 counts >350 cells/μl in the absence of HAART. Their magnitude of Gag-specific T cell responses was generally higher, more stable and the individuals peptides recognized were more conserved than acutely infected women. Furthermore, T cell recognition of p24 contributed >75% to the overall magnitude of Gag throughout the study and there was a significant inverse correlation between magnitude and breadth of p24 and viral load.

Five of the 9 LTNPs (56%) studied longitudinally recognized peptides which could harbor HLA-B*57/B*58/B*27-restricted epitopes: IW9 (Gag 36; NY219 and NY94), KF11 (Gag 40; NY99 and NY100) and KK10 (Gag 65; NY234) which have all previously been associated with LTNPs (Kaslow *et al.*; 1996; Migueles *et al*., 2000; Navis *et al*., 2007; Kiepiela *et al*., 2007; predicted from the Los Alamos Data base, http://www.hiv.lanl.gov/content/hiv-db/ELF/epitope analyzer.html; accessed 10 January 2011). The LTNPs in this study that recognized these protective epitopes are likely to have these protective HLA alleles (although HLA typing was not performed) and also had higher CD4 counts (although not significantly) compared to those who did not recognize these protective epitopes. All these findings suggest that targeting of Gag, especially the conserved p24 region, is a good correlate of protection against disease progression.

Role of HLA in HIV disease progression

Studies have shown the importance of certain HLA class I alleles, particularly HLA-B, in determining the rate of disease progression (Kiepiela *et al*., 2004). HLA-B*57/B*58 is overrepresented in LTNPs and certain HLA-B alleles are shown to be beneficial and others to be disadvantageous to the host (Goulder and Watkins, 2008). HLA-B57*/B*58 and B*27 are protective alleles whereas HLA-B*35 is associated with rapid disease progression. A major limitation of this study was the fact that the HLA type of the women enrolled in this study was not known (although HLA types were predicted based on peptides recognized).

Limitations of this study

Although there was a narrower response during acute infection, general statements of the regions and individual peptides targeted should be treated with caution because of the small number of samples and the lack of longitudinal samples. In addition, the ELISPOT assay is the most cost effective quantitative method for mapping epitope responses in HIV, this method generally allows only a single function to be measured at any time and IFNγ was measured in this study. While IFNγ has proven to be a useful surrogate for measuring CTL responses to HIV, other cytokines or T cell functions may be important in controlling HIV (Streeck *et al.*, 2009) and polyfunctional T cell responses have been implicated in control (Betts *et al*., 2006). In addition, the use of autologous virus sequence rather than consensus C peptides in this study may have enhanced the detection of HIV-specific T cell responses. A previous study by Altfeld *et al.* (2003) showed that p24-specific T cell responses were slightly higher in magnitude (although not significantly) when peptides based on autologous virus sequences were used compared with consensus peptides. Unlike flow cytometry and intracellular cytokine staining, the ELISPOT assay does not allow differentiation of the T cell subset responding to a particular peptide. ELISPOT approaches that detect two analytes have been developed that could overcome some of these limitation. Alternatively, multi-colour flow cytometry could be used in future studies to simultaneously measure multiple functions of individual T cell subsets.

Conclusions

In conclusion, HIV Gag- and Nef-specific T cell responses were clearly detectable in the majority of acutely HIV-infected women included in this study. In acutely infected women, HIV-specific responses were initially directed against a narrower array of peptides. Both the number of peptides as well as the cumulative magnitude of responses to both Gag and Nef increased over time in each woman. Throughout the first year of infection, responses to Gag and Nef were not conserved in women followed from acute infection but some peptide-specific responses were lost while others were gained, possibly reflecting loss of immunodominant responses to certain HIV epitopes because of CTL-induced immune escape in the circulating virus. In contrast, in LTNPs, T cell responses were predominantly against the highly conserved

p24 region of Gag and specific peptides recognized over approximately 18 months of follow up were remarkably stable. Few studies have investigated the evolution of T cell immune responses to HIV during subtype C infection and the role of responses mounted during acute infection in HIV subtype C pathogenesis. Although this study focused on acute responses in only a small group of subtype C infected women living in Sub-Saharan Africa, the findings presented in this dissertation suggest that early HIV-specific responses following infection fluctuate over time with emergence of new immunodominant specificities possibly reflecting escape of the circulating virus from immune pressure. In contrast, HIV-specific responses in LTNPs were remarkably stable over time.

Appendices

Appendices

Page

Appendix 1

Establishment of quality control PBMCs reactive with CEF peptides to include on each plate and in each ELISPOT assay

For quality assurance and in order to evaluate inter-assay and/or inter-plate variability in ELSIPOT, a single batch of PBMCs obtained from the Western Province Blood Transfusion Service was isolated and stored in ~10 identical aliquots in liquid nitrogen. In order to establish this reference lot of PBMCs, 20 HIV negative white cell buffy packs were obtained and screened for responsiveness to CEF peptides from the Western Province Blood Transfusion Service. White cell buffy packs are the cellular fraction of whole blood discarded by the Blood Transfusion Service after packed red blood cells have been removed. From 1 donor buffy pack, ~10^8 viable PBMCs can be obtained and frozen down in liquid nitrogen in aliquots of 10^7 cells per vial for subsequent experiments. PBMCs from these 20 uninfected donors were screened for responses to CEF peptides using the ELISPOT method described in Chapter 2 (section 2.12). Two donors were identified as having the highest magnitude CEF-specific responses: WP5101 with a mean of 1447 SFU/10^6 cells and WP1425 with a mean of 549 SFU/10^6 cells.

To determine the range of SFUs (including standard deviations) that would constitute a successful ELISPOT, replicates of 20 ELISPOT wells containing cells stimulated with CEF peptides from each of the selected donors were included in follow-up ELISPOT (shown in Figures A1 and A2).

The mean SFUs (including standard deviations) were calculated from these 20 replicates. From the results shown in Figures A1 and A2, the range of SFUs acceptable for WP 5105 was 970 -1924 and for WP 1425 was 289 – 809 per 10^6 PBMC. For the detection of inter-assay variability in this study, the response to CEF peptides had to fall within these ranges.

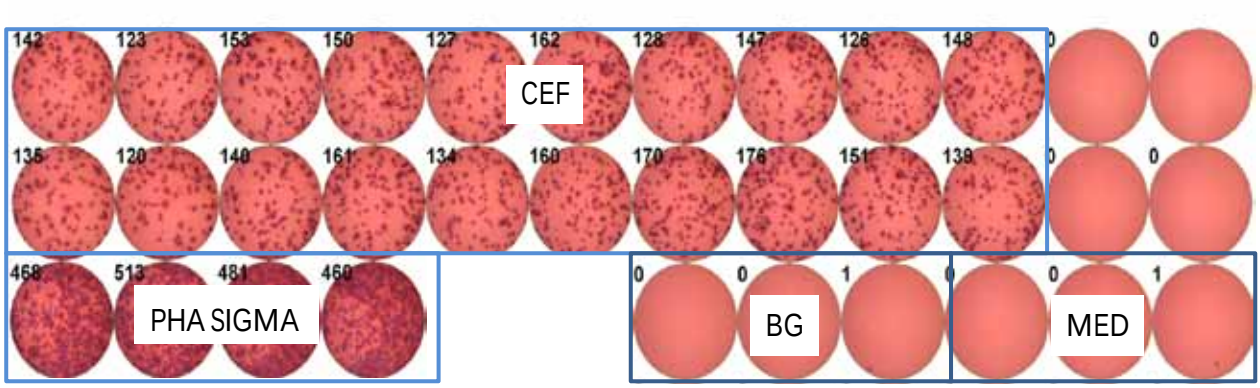


Figure A1 PBMC from WP5101 stimulated with CEF peptides to establish SFU ranges for quality control. The mean SFU and 3x Standard deviation was calculated to determine the range which constituted a successful ELISPOT. The range was 970 -1924 SFU per 10^6 cells.

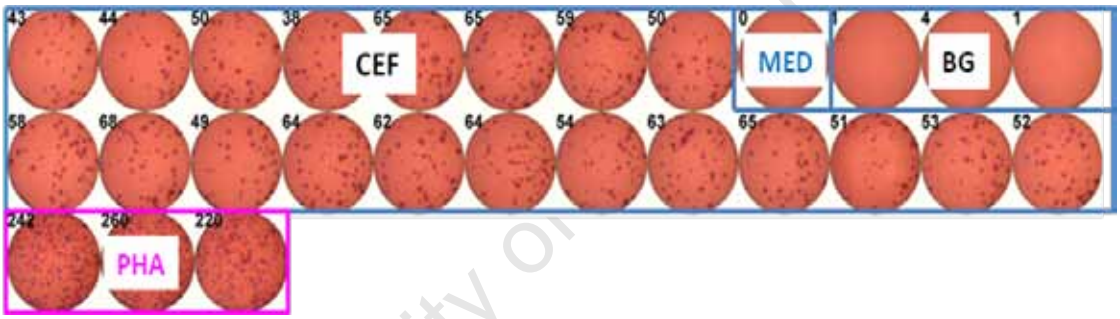


Figure A2 PBMC from WP1425 stimulated with CEF peptides to establish SFU ranges for quality control. The mean SFU and 3x Standard deviation was calculated to determine the range which constituted a successful ELISPOT. The range was 289 – 809 SFU per 10^6 cells.

In this study, PBMC from both donors were used as CEF controls. PBMC and CEF were plated in triplicate as described in Chapter 2 section 2.12. Eleven plates were used in the study which included control WP5105 (Figure A3 A) and 22 plates which included WP1425 as control (Figure A3 B). 10/11 (91%) assays for WP5105 were within the recommended range and one plate was slightly above the upper SFU range, although the result for that assay (plate 9) was accepted. All the assays which included WP1425 were within the recommended range.

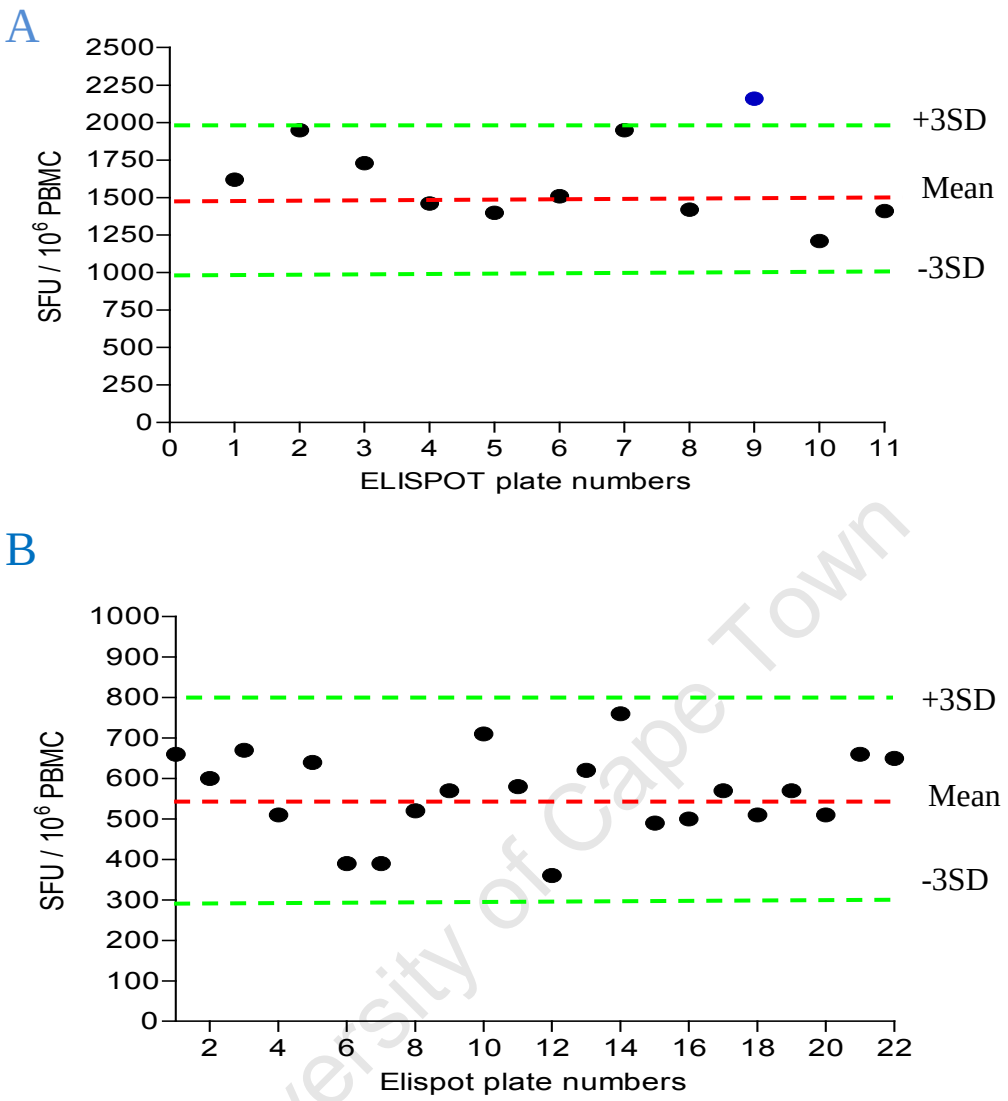


Figure A3 Range of inter assay CEF control obtained by ELISPOT. The red line is the mean and the green lines are the standard deviations (upper and lower) as calculated. **A**: In this study, PBMC from donor WP5105 were included in eleven assays and each spot which matched the calculated SFU per 10^6 PBMC, was plotted and checked if the range was maintained between (Figure A1) 970 -1924 SFU per 10^6 cells to monitor inter assay variability. **B:** In this study, PBMC from donor WP1425 were included in 22 assays and each spot which matched the calculated SFU per 10^6 PBMC was plotted and checked if the range was maintained between (Figure A2) 289 – 809 SFU per 10^6 cells to monitor inter assay variability.

Appendix 2

Optimization of PHA concentration for ELISPOT

PHA is a mitogen which reacts with and stimulates most mammalian cells. A comparative study was initially done to compare the ability of PHA (Sigma) to two different manufacturers to stimulate IFN-γ production in an ELISPOT assay. PHA was purchased from Sigma (Sigma-Aldrich, USA), Fluka (Buchs Switzerland) and Remel (Remel Europe Ltd.). PBMC from a HIV negative donor from Western Province Blood Transfusion Services (Pinelands, Cape Town) was stimulated with these three different PHAs at comparable concentrations; 16 and 24µg/ml (Sigma and Remel) and 20 and 40 µg/ml (Fluka). PHA from Remel and Fluka were previously used in our laboratory at these concentrations; and it is known from our experience that PHA from Fluka yields a lower SFU than PHA (Remel). Therefore, an initial concentration for PHA (Sigma) was selected that was similar to Remel. Results are shown in Figure B1. PBMCs were plated at 50 µl/well at a concentration of 2 $x10^6$/ml. From these experiments, no significant differences were observed in SFU/10^6 PBMCs when PHAs from Sigma or Remel were used but responses obtained using PHA from Sigma were significantly higher than those obtained using Fluka (p = 0.03; Figure B2). The current PHA from Sigma was at the concentration which yielded the highest response (16 µg /ml) was used for all subsequent experiments as the positive control.

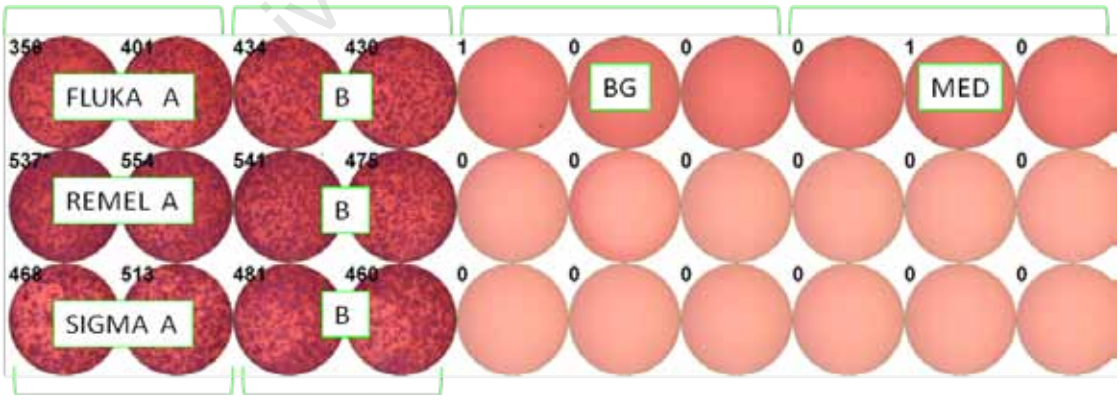


Figure B1 Comparison of IFN-γ responses generated using increasing concentrations of PHA from Fluka, Remel and Sigma. Row 1: Duplicates of PHA (Fluka; A= 20µg /ml B=40µg /ml). Row2: Duplicates of PHA (Remel; A=16 µg /ml and B=24µg /ml). Row 3: Duplicates of PHA (Sigma; A=16 µg /ml and B=24µg /ml). Included in this assay were triplicates of background and medium wells (Row1).

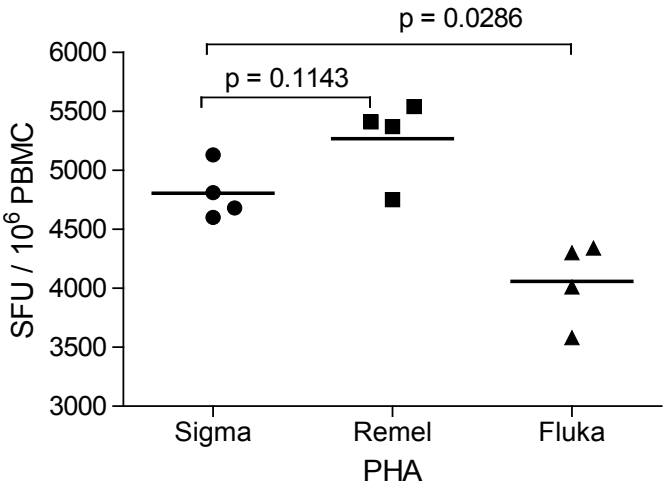


Figure B2 Comparison of IFN-γ responses generated using increasing concentrations of PHA from Fluka, Remel and Sigma. Each dot (Sigma, 16 µg /ml and 24µg /ml)), square (Remel, 16µg /ml and 24µg /ml) and triangle (Fluka, 20µg/ ml and 40µg /ml) represents matched measurements of SFU per 10^6 PBMC and the three concentrations.

Appendix 3

Quality control between different batches of HIV Gag peptides by ELISPOT

Two different batches of HIV Gag peptides were used in this study and a comparative ELISPOT was performed to compare the efficacy of each peptide batch to stimulate IFN-γ production by ELISPOT. PBMCs from two HIV-1 positive donors were stimulated with the new Gag pools 1 - 5 prepared exclusively for this study and with the current Gag pools 1-5 routinely used in the laboratory. Figure C1 shows the comparative response magnitudes obtained from 2 donors. According to the results, IFN-γ responses that were generated using the new set of Gag pools significantly correlated to the responses produced by the existing Gag (p = 0.01, Spearman Rho = 0.76; Figure C2).

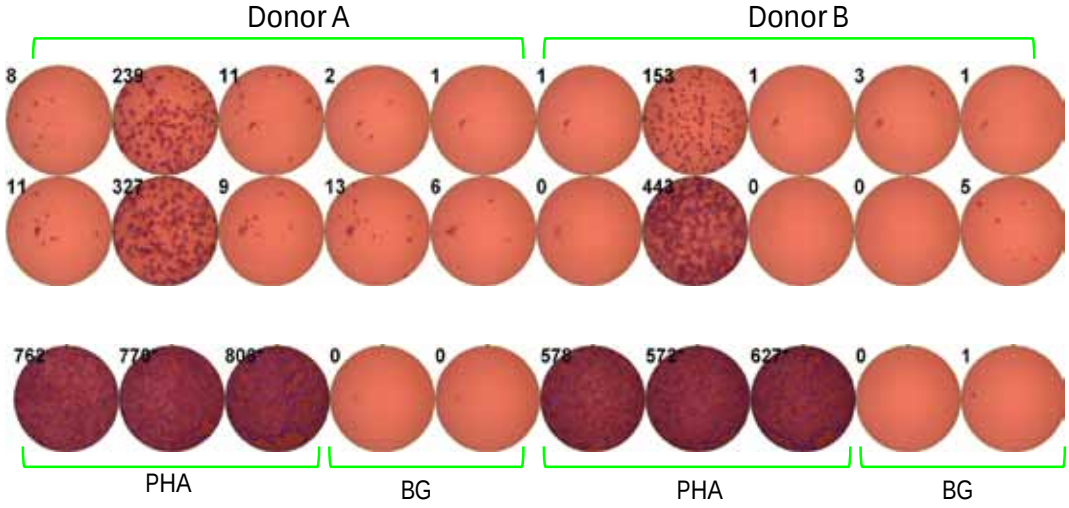


Figure C1 Comparison of different batches of HIV Gag peptides to stimulate IFNγ responses by ELISPOT. Row 1 represents PBMCs from Donor A and B with current Gag pools 1-5. Row two represents PBMC from Donor A and B with the new set of Gag 1-5 Pools. Included are PHA and background controls (Row 3).

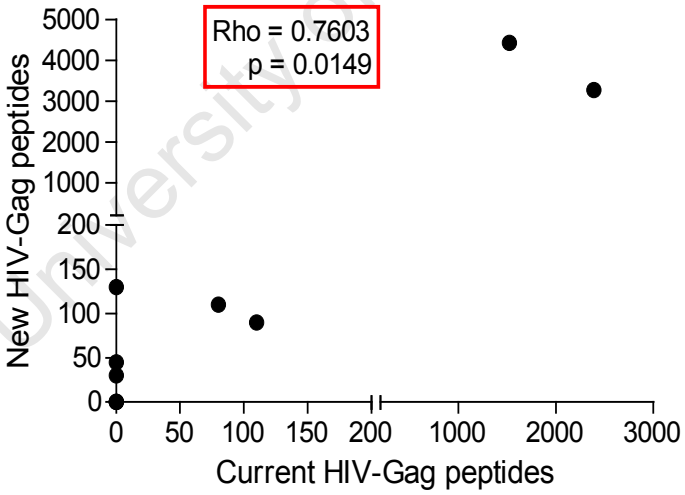


Figure C2 Association between two different HIV Gag peptide pools. Each dot represents matched measurement of IFN-γ responses (SFU/10^6 PBMC) of current set and new set of HIV- Gag pools 1-5. Mann-Whitney U tests were applied to compare these non-parametric variables and p values <0.05 were accepted as significant.

Appendix 4

Kinetics of HIV plasma viral load and CD4 T cell counts during the course of HIV infection

HIV RNA viral load (Tables A1 for acute infection and A3 for LTNPs) and CD4 T cell counts (Table A2 for acute infection and A4 for LTNPs) measurements of seven women acutely infected with HIV and nine LTNPs were monitored throughout the duration of this study.

Table A1 Kinetics of plasma HIV viral load during the course of acute HIV infection

		HIV viral loads (RNA copies/ml plasma) at each time point post infection (months)									
PID	**Incident**	**1**	**2**	**3**	**4**	**6**	**9**	**12**	**18**	**24**	**30**
C001	-	9430	4460	2020	6980	4890	4440	2430	4230	20200	18400
C002	7480	-	-	4510	7410	-	-	-	-	-	-
C004	11000	-	-	87600	102000	14400	182000	87600	134000	42800	-
C007	228000	195000	196000	599000	-	-	-	-	-	-	-
C008	16900	13900	< 50	6550	15500	< 50	10500	41600	7820	-	-
B349	6400	-	-	-	-	-	-	-	-	-	-
B420	31200	-	-	-	-	-	-	-	-	-	-
Median	13950	13900	4460	6550	11455	4890	10500	41600	7820	31500	-
Min	6400	9430	<50	2020	6980	<50	4440	2430	4230	20200	-
Max	228000	195000	196000	599000	102000	14400	182000	87600	134000	42800	-

Table A2 CD4 decline during the course of acute HIV infection

		CD4 T cell counts at each time point post infection (months)									
PID	**Incident**	**1**	**2**	**3**	**4**	**6**	**9**	**12**	**18**	**24**	**30**
C001	548	747	708	759	555	543	550	615	651	579	521
C002	548	-	-	592	581	-	-	-	-	-	-
C004	867	-	-	829	495	865	692	675	471	554	-
C007	177	383	373	274	-	-	-	-	-	-	-
C008	787	818	720	776	925	703	567	537	505	-	-
B349	305	-	-	-	-	-	-	-	-	-	-
B420	298	-	-	-	-	-	-	-	-	-	-
Mean	504	649	600	646	639	704	603	609	542	567	-
SD	260	233	197	226	194	161	78	69	96	18	-

Table A3 Kinetics of plasma HIV load during the course of LTNP infection.

	Plasma HIV loads (RNA copies/ml) at each time point during chronic infection (months)										
PID	66	72	78	84	90	96	102	108	114	120	132
NY010	<50	-	<50	-	<50	-	-	-	-	-	-
NY040	-	-	-	7200	3100	-	-	32000	-	-	-
NY064	-	-	-	930	2900	2400	1700	-	-	-	-
NY094	-	1700	1900	-	4400	-	-	-	-	-	-
NY099	-	10000	8500	-	-	16000	-	-	-	-	-
NY100	-	-	-	-	53000	56000	-	-	-	-	-
NY219	16000	20000	94	-	-	-	-	-	-	-	-
NY234	-	2 600	-	-	17 000	-	-	-	-	-	-
NY292	-	-	-	-	-	-	-	< 50	64	75	370
Median	8025	6300	997	4065	3750	16000	-	16025	-	-	-
Min	<50	1700	<50	930	<50	2400	-	<50	-	-	-
Max	16000	20000	8500	7200	53000	56000	-	32000	-	-	-

Table A4 Kinetics of CD4 counts during the course of LTNP HIV infection.

	CD4 T cell counts (cell/µl) at each time point during chronic infection (months)										
PID	66	72	78	84	90	96	102	108	114	120	132
NY010	334	-	422	-	521	-	-	-	-	-	-
NY040	-	-	-	322	396	-	-	300	-	-	-
NY064	-	-	-	383	443	324	790	-	-	-	-
NY094	-	465	427	-	428	-	-	-	-	-	-
NY099	-	525	630	-	-	383	-	-	-	-	-
NY100	-	-	-	-	778	599	-	-	-	-	-
NY219	551	465	265	-	-	-	-	-	-	-	-
NY234	-	680	-	-	775	-	-	-	-	-	-
NY292	-	-	-	-	-	-	-	529	536	574	605
Mean	443	534	436	353	557	435	-	415	-	-	-
SD	153	102	150	43	175	145	-	162	-	-	-

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